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OBSERVATIONS ON THE METABOLISM OF NORMAL AND ARSENIC-RESISTANT TRYPANOSOMA GAMBIENSE

THEODOR von BRAND, ELEANOR JOHNSON TOBIE, BENJAMIN MEHLMAN AND EUGENE C. WEINBACH

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EIGHT FIGURES

Resistance of the parasites to arsenical drugs is of considerable practical importance in African trypanosomiasis since it interferes seriously with the chemotherapeutic control of the disease. The mechanism of arsenic resistance in trypanosomes has been studied from a primarily pharmacological or biological standpoint by Voegtlin, Dyer and Miller ('24), Murgatroyd and Yorke ('37), Yorke, Murgatroyd and Hawking ('31), Fulton and Yorke ('41), Eagle and Magnuson ('44), and others; *Trypanosoma equiperdum* and *T. rhodesiense* were used chiefly. Biochemical studies were done by Fulton and Christophers ('38) with *T. rhodesiense,* and Harvey ('48, '49) with *T. equiperdum* and *T. hippicum.* So far, *T. gambiense,* which in Africa readily develops arsenic resistance, has not been studied biochemically.

The aim of our study is to give data on (1) the type of carbohydrate metabolism present in *T. gambiense,* (2) the quantitative metabolic aspects of both a normal and an arsenic-resistant strain, and (3) the response of both types of trypanosomes to a variety of metabolic inhibitors. These last studies were done in order to shed some light on the question of whether or not arsenic resistance is accompanied by definite metabolic changes or is due solely to changes in permeability.

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MATERIALS AND METHODS

We had available for our studies a normal, arsenic-sensitive strain of *Trypanosoma gambiense* and a substrain rendered resistant to reduced tryparsamide,

This, according to a personal communication of Dr. F. Murgatroyd, is the same compound used by Yorke, Murgatroyd and Hawking ('32) in their studies on *T. rhodesiense,* a point we want to emphasize, because apparently some doubts exist in the minds of organic chemists as to what compound was called reduced tryparsamide by Yorke, et al. We received the compound through the courtesy of Dr. S. Rieveschl, Parke, Davis, and Co., in whose laboratory it was synthesized. The method employed in producing arsenic resistance was essentially the method of *in vitro* exposure to successively increased concentrations of reduced tryparsamide developed by Yorke, et al. ('32). This phase of the problem and the purely biological characteristics of the strain will be discussed in a separate paper. It is sufficient to mention here that the highest concentration of reduced tryparsamide initially survived was 1:3,200,000, while after the 25th exposure a concentration of 1:3125 was nonlethal. Since initially the next concentration tested was 1:800,000, which proved lethal, and since no intermediate concentrations were tested, it cannot be concluded that the increase in resistance was a thousand-fold. Actually, the respiration studies related in a later section indicate an increase of resistance of the order of magnitude of 600.

The arsenic-sensitive strain is hereinafter designated as "normal strain." The terms "resistant strain 8th, 13th, or 25th exposure" indicate that the trypanosomes had undergone 8, 13, or 25 exposures *in vitro* to reduced tryparsamide. They do not, of course, mean that the trypanosomes were used im-

mediately after these respective exposures. The exposed flagellates were injected into fresh rats and the strains maintained by inoculation of infected blood into other rats and used at varying intervals thereafter as necessitated by the experiments without being reexposed to the drug. This procedure was permissible because arsenic resistance of trypanosomes, once established, is an extremely stable character (Murgatroyd and Yorke, '37; Fulton and Yorke, '41). As a safety measure we tested towards the end of our experiments (about 6 months after the last *in vitro* exposure) the sensitivity of both the normal strain and the resistant strain, 25th exposure, to reduced tryparsamide and found both entirely unchanged. This proves that the resistant strain had maintained its resistance, since the tolerance to the high concentration can obviously not have been due to a deterioration of the compound. This point is stressed because initially there was some doubt expressed by chemists as to whether reduced tryparsamide is a stable compound.

The manometric methods were the same as those used in our previous studies, and reference is made to the papers by von Brand and Johnson ('47), von Brand and Tobie ('48), and von Brand, Tobie and Mehlman ('50). All values given below are corrected for the endogenous oxygen consumption of blood or serum respectively.

The glucose determinations were carried out by the method of Hagedorn and Jensen ('23). We found that the main metabolic endproduct of the trypanosomes, pyruvic acid, has a slight but measurable reducing effect on ferricyanide. A standard curve was established, and the glucose values were corrected both for this factor and the glycolysis of the blood. When serum was used as medium only the pyruvic acid correction proved necessary.

Pyruvic acid was determined by the method of Friedemann and Haugen ('43), and parallel experiments were done in some cases by means of Slavic and Michalec's ('49) modification. It is more specific for pyruvic acid since the interfering oxaloacetic and α-ketoglutaric acids are removed. The figures ar-

rived at in experiments with whole-blood medium are minimal values, since pyruvic acid has a tendency to disappear from blood (Bueding and Wortis, '40). It was not possible to introduce a valid correction, because of the shift in concentration of pyruvate occurring in our experiments. However, the error introduced cannot have been very large, since experiments carried out in serum gave only slightly, but nevertheless distinctly higher, pyruvate values.

The over-all metabolism of Trypanosoma gambiense

During the course of the present study, numerous experiments were performed both with the normal strain and with the drug-resistant strain, in which oxygen and sugar consumption, as well as pyruvate production, were determined simultaneously. In these experiments no inhibitors were used, and the medium consisted of either 0.2 ml 0.85% saline + 0.8 ml rabbit serum, or 0.2 ml 0.85% saline + 0.8 ml rat blood. In the serum experiments, the trypanosomes had been separated from the rat erythrocytes by centrifugation and the rabbit serum obviously contained a small admixture of rat serum. The trypanosome concentration in the serum experiments varied in most experiments between about 50 and 150 million per milliliter, in the blood experiments between about 30 and 80 million per milliliter.

A survey of these experiments, shown in table 1, indicates that the metabolic rate was significantly lowered in serum as compared to that observed in blood. The fact that the metabolic rate of trypanosomes is relatively low in blood-free medium had already previously been described by Marshall ('48) for *T. evansi,* and by Moulder ('48) who found the respiration of *T. lewisi* in serum to be 74% of that in whole blood. Our data indicate that the percentage reduction was approximately the same in the normal strain and the drug-exposed strain, both at a time when drug resistance had not yet developed (8th and 13th exposure) and at a time when drug resistance had reached its maximum (25th exposure). There is a strong suggestion that the over-all metabolism of the highly

TABLE 1

Quantitative aspects of metabolism of normal and arsenic-resistant Trypanosoma gambiense per 100 million per 1 hour, 37°C.

STRAIN	MEDIUM	NUMBER OF DETERMINATIONS	GLUCOSE CONSUMED	PYRUVATE PRODUCED	OXYGEN CONSUMED	MOLAR RATIO		
						Glucose	Pyruvate	Oxygen
			mg	*mg*	*mm³*			
Normal	Serum	36	0.78 ± 0.04	0.81 ± 0.04	113 ± 4.9	1	2.1	1.2
Resistant 13th exposure	Serum	6	0.85 ± 0.06	0.80 ± 0.04	119 ± 7.6	1	1.9	1.1
Resistant 25th exposure	Serum	30	0.63 ± 0.04	0.66 ± 0.03	93 ± 6.1	1	2.1	1.2
Normal	Blood	32	1.43 ± 0.06	1.19 ± 0.05	161 ± 4.4	1	1.7	1.0
Resistant 8th exposure	Blood	29	1.53 ± 0.08	1.38 ± 0.05	171 ± 6.1	1	1.8	0.9
Resistant 25th exposure	Blood	49	1.18 ± 0.04	0.99 ± 0.03	163 ± 5.6	1	1.7	1.2

resistant strain was somewhat lower than that of the normal strain, or the strain before it developed full resistance. The differences are most pronounced and apparently significant in regard to sugar consumption and pyruvate production, while much less developed, or even absent, in the case of oxygen consumption.

It is of interest to note that the molar ratio between glucose, pyruvate, and oxygen was in all cases fairly close to the proportion 1 : 2 : 1. While we cannot, of course, exclude the possibility that other endproducts may be formed in small amounts, we have definitely established pyruvic acid as the main endproduct. The method of Slavic and Michalec ('49) gave, within reasonable limits, the same results as that of Friedemann and Haugen ('43), establishing the above point beyond reasonable doubt. The somewhat smaller proportion of pyruvic acid in whole-blood medium compared with that found in serum is almost certainly due to a spontaneous disappearance of a certain fraction induced by the metabolism of the red cells.

Reaction to inhibitors

All the experiments summarized in this section were conducted in whole blood because it was felt essential to work under conditions allowing maximal respiratory activities. If this had not been done, a valid criticism would have been that the part of the respiration lost in serum could conceivably have reacted differently to inhibitors in the normal and the arsenic-resistant strains.

All experiments were conducted in identical fashion. They lasted 40 minutes and the temperature was 37°C. One noninhibited control and 7 different drug concentrations were used for the determination of the oxygen consumption, while glucose consumption and pyruvate production were determined in the noninhibited control and in two to three selected drug concentrations. With some inhibitors either glucose, pyruvate, or both could not be studied because the inhibiting compound interfered with the determinations. All experiments were repeated at least 4 times, and in some cases up to 10 times.

Two organic arsenicals, reduced tryparsamide and mapharsen, and one inorganic arsenical, sodium arsenite, were employed. The data presented in figure 1 indicate that after the 8th exposure to reduced tryparsamide no trace of arsenic resistance was evident. After the 25th exposure, on the contrary, resistance to reduced tryparsamide was extremely well developed, an evaluation of figure 1 reveals an approximate 600-fold increase in resistance, as indicated both by the rates

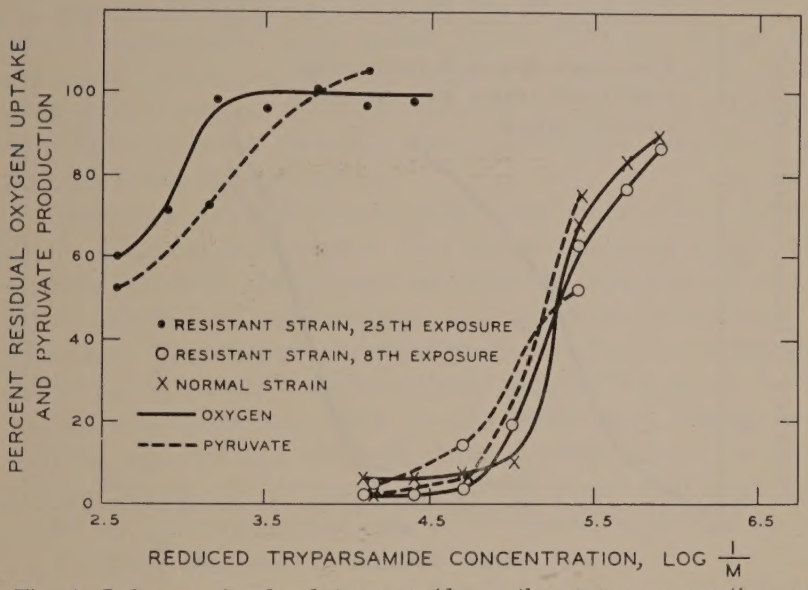

Fig. 1 Influence of reduced tryparsamide on the oxygen consumption and pyruvate production of normal and arsenic-resistant *Trypanosoma gambiense*.

of oxygen consumption and pyruvate production. Sugar determinations could not be done with this compound because the higher concentrations of the drug interfered with the determinations. The increase in resistance was much smaller, but still considerable, when mapharsen was used; it can be estimated to be about 30 times (fig. 2).

The picture offered by sodium arsenite (table 2) was somewhat different. A very small, but rather consistent increase in resistance was found in the strain that had undergone 25 ex-

posures to reduced tryparsamide, as indicated by the rates of oxygen consumption, while the phenomenon is hardly noticeable in respect to sugar consumption and pyruvate production. In this case a series of 10 experiments was done, and it appears probable that the difference between the nonresistant and the resistant 25th exposure strain is real, although the differences between both are not statistically significant at each concentration. This apparent increase in resistance to an inorganic

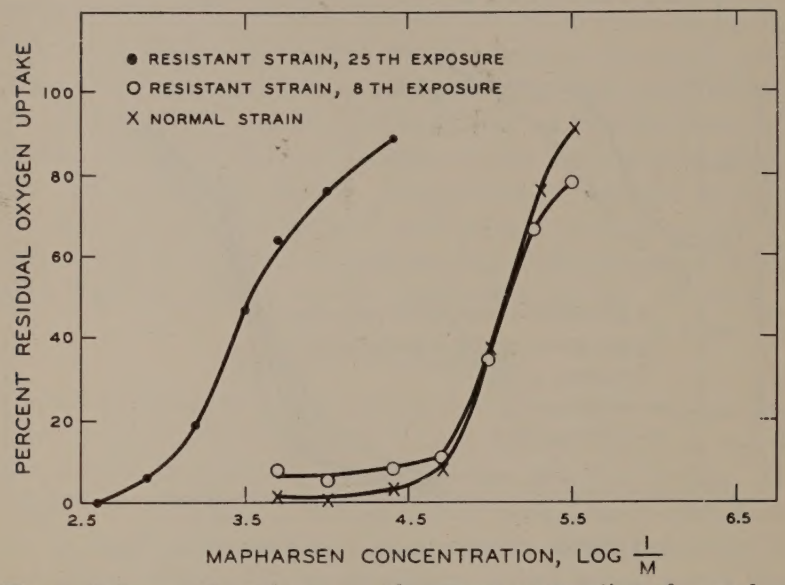

Fig. 2 Influence of mapharsen on the oxygen consumption of normal and arsenic-resistant *Trypanosoma gambiense*.

arsenical, although admittedly only very small, is nevertheless of theoretical interest and we shall return to this point in the discussion.

Arsenicals are usually considered as sulfhydryl inhibitors. Consequently it seemed of interest to test whether other types of sulfhydryl inhibitors would give the same result as arsenicals. Two such compounds, iodoacetate and iodoacetamide were tested. Table 2 makes it quite evident that the response was different. Both compounds were quite effective in inhibit-

ing the flagellates, but the highly arsenic-resistant strain did not show even the slightest increase in resistance to either compound. If any change at all took place, the arsenic-resistant strain was even slightly more inhibited by iodoacetate than the normal one.

Sodium fluoride, often a very potent inhibitor of glycolysis, was not very effective against *Trypanosoma gambiense,* only a concentration of M/100 inhibiting the flagellates by about 50%. No significant difference between the normal and arsenic-resistant strain could be detected. Borei ('45) has shown that the main, though not the only, point of attack of fluoride is the cytochrome system. Since the African trypanosomes lack the cytochrome system (Harvey, '49), the relative inefficiency of fluoride is not too surprising.

Cinnamate (table 2) showed little effect on either the normal or the drug-resistant strain. The highest concentrations used slightly inhibited the oxygen consumption, and a small stimulation of glucose consumption and pyruvate production is probable. The effect is fundamentally similar, but much less pronounced, than in some vertebrate tissues (Weinbach, Lowe, Frisell and Hellerman, '51). In the latter these effects have been interpreted as a reversal of the Pasteur effect. The relative ineffectiveness of cinnamate on organisms that show normally an almost maximal rate of aerobic glycolysis is understandable.

Malonate and fluoroacetate (table 2) did not significantly interfere with the metabolism of either the normal or the arsenic-resistant trypanosomes. The highest concentration used, M/100, may have inhibited somewhat the glucose consumption. But this effect was quite variable in different experiments and hence may not be real. Both compounds are known to interfere in many cases with reactions of the tricarboxylic acid cycle, which according to the present state of our knowledge, is absent in the bloodstream form of the African trypanosomes.

Cyanide and azide (figs. 3 and 4) exert their chief activity by interfering with heavy metal catalysis. *Trypanosoma gam-*

TABLE 2

Influence of some inhibitors on the metabolism of Trypanosoma gambiense

All metabolic processes are expressed in per cent of those shown by noninhibited controls, representing therefore residual processes. Most figures are averages of 4 experiments, but in the case of arsenite, of 10 experiments. The figures behind the ± signs are the standard errors of the mean.

O_2 = mm^3 O_2/100 million/1 hour; glucose = mg glucose/100 million/1 hour; pyruvate = mg pyruvic acid/million/1 hour.

Resistant, 8th = strain used after 8 exposures to reduced tryparsamide.

Resistant, 25th = strain used after 25 exposures to reduced tryparsamide.

Sodium arsenite

STRAIN	M/5,000			M/7,500	M/10,000			M/17,500		
	O_2	glucose	pyruvate	O_2	O_2	glucose	pyruvate	O_2	glucose	pyruvate
Normal	1 ± 0.8	13 ± 4.8	11 ± 4.3	3 ± 1.4	11 ± 2.0			36 ± 3.4	49 ± 7.1	48 ± 2.4
Resistant, 8th	0 ± 0.0	13 ± 6.8	7 ± 2.0	2 ± 1.2	11 ± 4.0			48 ± 2.6	67 ± 13.6	53 ± 3.3
Resistant, 25th	3 ± 1.3	23 ± 3.7	13 ± 1.5	13 ± 2.8	28 ± 3.8			49 ± 2.7	62 ± 3.8	67 ± 4.1

	M/25,000			M/50,000			M/100,000
	O_2	glucose	pyruvate	O_2	glucose	pyruvate	O_2
Normal	61 ± 3.2			76 ± 3.1	80 ± 4.7	87 ± 2.1	89 ± 2.6
Resistant, 8th	60 ± 14.0			84 ± 4.3	94 ± 13.9	89 ± 2.2	98 ± 2.0
Resistant, 25th	65 ± 2.4			89 ± 2.4	89 ± 3.4	92 ± 2.6	96 ± 2.7

Iodoacetate

STRAIN	M/1,000			M/5,000	M/7,500			M/10,000		
	O_2	glucose	pyruvate	O_2	O_2	glucose	pyruvate	O_2	glucose	pyruvate
Normal	9 ± 5.7	13 ± 5.9	0 ± 0.0	34 ± 8.0	52 ± 5.8			58 ± 5.6	74 ± 8.5	64 ± 3.8
Resistant, 8th	8 ± 3.3	7 ± 4.1	0 ± 0.0	27 ± 2.9	45 ± 9.8			68 ± 8.7	61 ± 11.3	58 ± 5.6
Resistant, 25th	0 ± 0.0	0 ± 0.0	0 ± 0.0	13 ± 3.9	35 ± 3.9			47 ± 4.7		

	M/25,000			M/50,000			M/75,000
	O_2	glucose	pyruvate	O_2	glucose	pyruvate	O_2
Normal	88 ± 6.7			81 ± 10.4	122 ± 15.0	106 ± 6.9	95 ± 2.5
Resistant, 8th	96 ± 4.0			97 ± 1.3	99 ± 12.1	101 ± 2.3	96 ± 1.3
Resistant, 25th	82 ± 8.0			89 ± 4.4	83 ± 7.6	86 ± 9.0	90 ± 10.7

STRAIN	M/200			M/400	M/600			M/1,000		
	O_2	glucose	pyruvate	O_2	O_2	glucose	pyruvate	O_2	glucose	pyruvate
Normal	7 ± 1.9	5 ± 4.5		53 ± 4.4	74 ± 5.5	61 ± 11.5		87 ± 5.7		
Resistant, 8th	4 ± 2.2	4 ± 3.0	3 ± 3.0	50 ± 4.5	71 ± 0.6	73 ± 6.9	76 ± 2.9	80 ± 1.4		
Resistant, 25th	12 ± 5.1	0 ± 0.0	0 ± 0.0	48 ± 5.3	76 ± 1.5	73 ± 5.4	78 ± 4.9	78 ± 2.7		

	M/2,000		
	O_2	glucose	pyruvate
Normal	83 ± 2.7	86 ± 20.7	
Resistant, 8th	92 ± 6.7	86 ± 8.4	101 ± 1.5
Resistant, 25th	91 ± 4.4	87 ± 11.0	93 ± 5.1

Sodium fluoride

STRAIN	M/100			M/200	M/500			M/1,000		
	O_2	glucose	pyruvate	O_2	O_2	glucose	pyruvate	O_2	glucose	pyruvate
Normal	52 ± 4.8	48 ± 8.7	62 ± 1.0	75 ± 6.6	87 ± 5.3	98 ± 11.0	101 ± 0.8	94 ± 7.0		
Resistant, 8th	51 ± 3.3	28 ± 7.3	44 ± 2.6	68 ± 3.0	87 ± 3.8	88 ± 10.0	100 ± 4.9	94 ± 5.9		
Resistant, 25th	51 ± 4.8	26 ± 2.7	43 ± 2.9	80 ± 6.5	93 ± 7.9	88 ± 9.7	98 ± 2.7	85 ± 8.7		

	M/5,000			M/10,000			M/50,000
	O_2	glucose	pyruvate	O_2	glucose	pyruvate	O_2
Normal	94 ± 7.0	99 ± 4.2	104 ± 2.0	95 ± 3.1			98 ± 1.7
Resistant, 8th	95 ± 6.4	96 ± 18.1	108 ± 1.7	101 ± 8.1			92 ± 4.6
Resistant, 25th	97 ± 6.4	96 ± 7.4	104 ± 1.7	93 ± 14.9			98 ± 12.3

Cinnamate

STRAIN	M/50			M/100	M/250			M/500		
	O_2	glucose	pyruvate	O_2	O_2	glucose	pyruvate	O_2	glucose	pyruvate
Normal	80 ± 10.2	102 ± 7.5	86 ± 7.0	87 ± 6.8	92 ± 7.4	108 ± 8.0	113 ± 1.0	100 ± 6.8		
Resistant, 8th	85 ± 3.7	109 ± 17.9	83 ± 4.0	88 ± 0.9	99 ± 1.6	113 ± 9.1	109 ± 2.3	102 ± 3.6		
Resistant, 25th	80 ± 3.3	112 ± 1.2	95 ± 3.6	83 ± 2.9	95 ± 3.7	114 ± 0.5	110 ± 1.9	88 ± 6.4		

	M/1,000			M/5,000			M/10,000
	O_2	glucose	pyruvate	O_2	glucose	pyruvate	O_2
Normal	102 ± 9.3			96 ± 6.1	102 ± 14.5	102 ± 0.7	96 ± 8.5
Resistant, 8th	108 ± 3.4			102 ± 2.5	107 ± 1.7	121 ± 6.1	101 ± 3.7
Resistant, 25th	97 ± 3.0			94 ± 4.7	108 ± 3.7	105 ± 2.7	95 ± 6.0

TABLE 2 *(continued)*

Influence of some inhibitors on the metabolism of Trypanosoma gambiense

All metabolic processes are expressed in per cent of those shown by noninhibited controls, representing therefore residual processes. Most figures are averages of 4 experiments, but in the case of arsenite, of 10 experiments. The figures behind the ± signs are the standard errors of the mean.

O_2 = mm^3 O_2/100 million/1 hour; glucose = mg glucose/100 million/1 hour; pyruvate = mg pyruvic acid/million/1 hour.

Resistant, 8th = strain used after 8 exposures to reduced tryparsamide.

Resistant, 25th = strain used after 25 exposures to reduced tryparsamide.

Malonate

STRAIN	M/100			M/200	M/400			M/600		
	O_2	glucose	pyruvate	O_2	O_2	glucose	pyruvate	O_2	glucose	pyruvate
Normal	95 ± 3.3	57 ± 14.6	102 ± 7.7	92 ± 3.9	98 ± 1.5	94 ± 11.4	105 ± 7.6	99 ± 4.1		
Resistant, 8th	94 ± 1.9	77 ± 11.9	95 ± 1.9	90 ± 1.6	94 ± 2.0	95 ± 6.0	100 ± 2.1	96 ± 1.9		
Resistant, 25th	96 ± 2.3	63 ± 10.7	105 ± 3.7	95 ± 3.3	92 ± 2.8	82 ± 8.1	99 ± 5.0	93 ± 3.5		

	M/1,000			M/2,000			M/5,000
	O_2	glucose	pyruvate	O_2	glucose	pyruvate	O_2
Normal	94 ± 3.5	99 ± 10.6	101 ± 6.5	93 ± 3.0			94 ± 3.7
Resistant, 8th	100 ± 3.3	96 ± 9.3	99 ± 10.6	101 ± 3.5			98 ± 3.0
Resistant, 25th	89 ± 3.5	93 ± 7.1	97 ± 8.4	89 ± 9.1			93 ± 5.0

Fluoroacetate

STRAIN	M/100			M/200	M/400			M/600		
	O_2	glucose	pyruvate	O_2	O_2	glucose	pyruvate	O_2	glucose	pyruvate
Normal	102 ± 3.6	133 ± 20.4	110 ± 1.6	96 ± 1.2	98 ± 4.0	123 ± 8.1	108 ± 2.6	97 ± 5.8		
Resistant, 8th	96 ± 0.9	117 ± 8.7	103 ± 2.8	94 ± 0.6	95 ± 1.0	115 ± 9.6	106 ± 1.6	94 ± 1.3		
Resistant, 25th	98 ± 1.9	103 ± 4.1	103 ± 1.1	95 ± 1.1	94 ± 3.3	107 ± 5.2	102 ± 1.6	89 ± 5.4		

	M/1,000			M/2.000			M/5,000
	O_2	glucose	pyruvate	O_2	glucose	pyruvate	O_2
Normal	100 ± 3.1	99 ± 9.4	104 ± 2.0	99 ± 3.4			98 ± 2.8
Resistant, 8th	94 ± 5.6	113 ± 16.0	104 ± 2.2	96 ± 5.1			96 ± 2.8
Resistant, 25th	89 ± 4.5	104 ± 4.9	98 ± 2.5	96 ± 4.5			91 ± 5.4

biense, like all African trypanosomes, is normally quite insensitive to cyanide and often even stimulated (von Brand and Tobie, '48). The same response was obtained in the present series. Our data contain a suggestion that arsenic resistance is accompanied by a slight development of cyanide sensitivity. The differences are small and not entirely significant from a

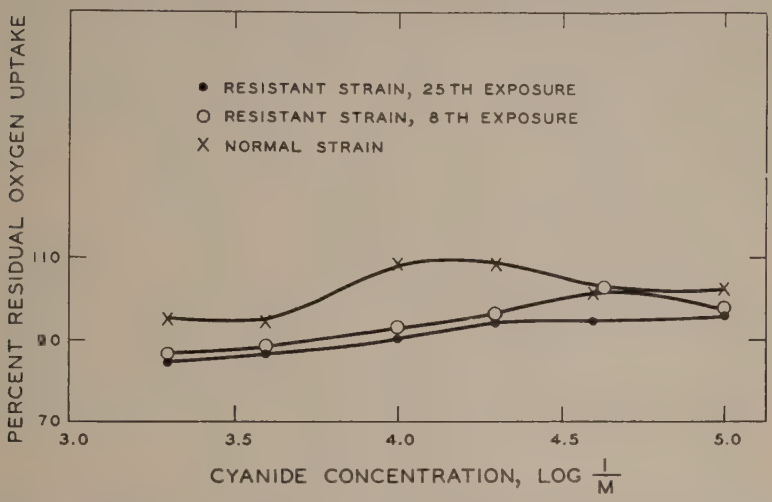

Fig. 3 Influence of cyanide on the oxygen consumption of normal and arsenic-resistant *Trypanosoma gambiense.*

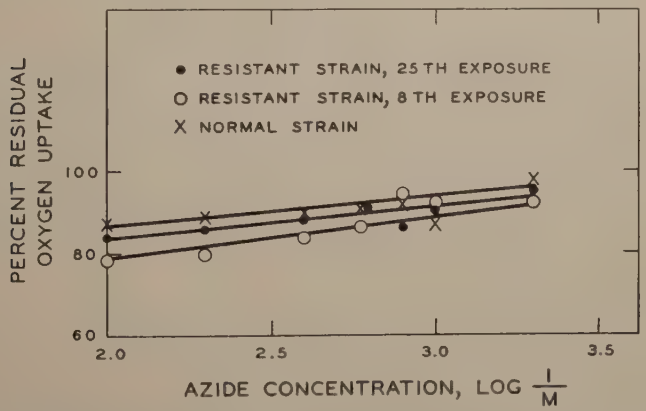

Fig. 4 Influence of azide on the oxygen consumption of normal and arsenic-resistant *Trypanosoma gambiense.*

statistical standpoint. However, the lower rate of oxygen consumption at every concentration tested, does suggest that the phenomenon is real. Azide, not tested heretofore on *T. gambiense*, produced a very slight inhibition of respiration; the straight-line relationship between residual respiration and azide concentration indicates clearly that some inhibition was induced, but no significant difference between normal and drug-resistant trypanosomes existed. It would appear then

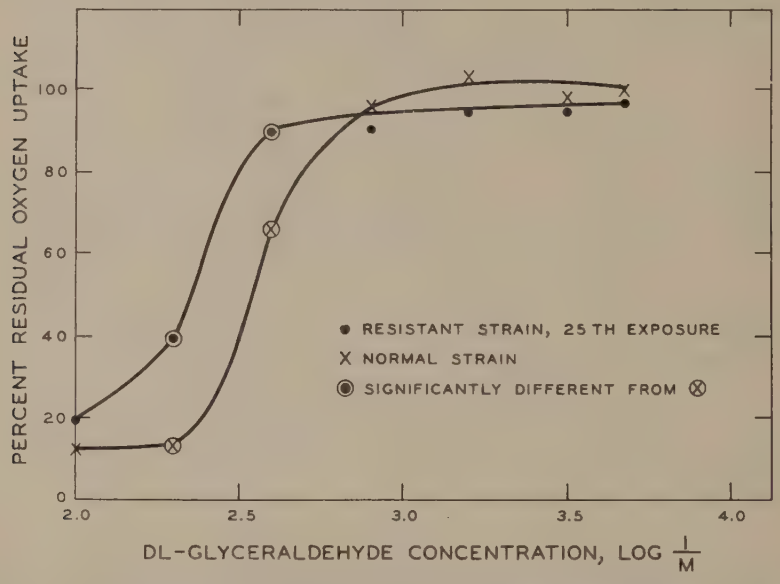

Fig. 5 Influence of D,L-glyceraldehyde on the oxygen consumption of normal and arsenic-resistant *Trypanosoma gambiense*.

that the response of *Trypanosoma gambiense* to cyanide and azide is not exactly parallel. This is not too surprising, since some other biological objects show even more pronounced differences (Stannard, '39).

D,L-glyceraldehyde (fig. 5), by combination of its L-isomer with triosephosphate and formation of L-sorbose-1-phosphate, inhibits hexokinase activity (Lardy, Wiebelhaus and Mann, '50). The compound rather effectively inhibited the oxygen consumption of *Trypanosoma gambiense* at concentrations

comparable to those effective in mammalian tissues. It is of interest to note that the arsenic-resistant strain showed a slight, but definitely significant decrease in sensitivity to the compound.

Three nitrofurans were tested, nitrofurazone, compound NF. 114 (5-nitro-2-furaldehyde trimethyl-ammonium acethydrazone chloride) and compound NF. 185 (5-nitro-2-furaldehyde 4-(3-diethyl-aminopropyl) semicarbazone hydrochloride). These compounds were received through the courtesy of Dr. W. B. Stillman, Eaton Laboratories. Nitrofurazone, because

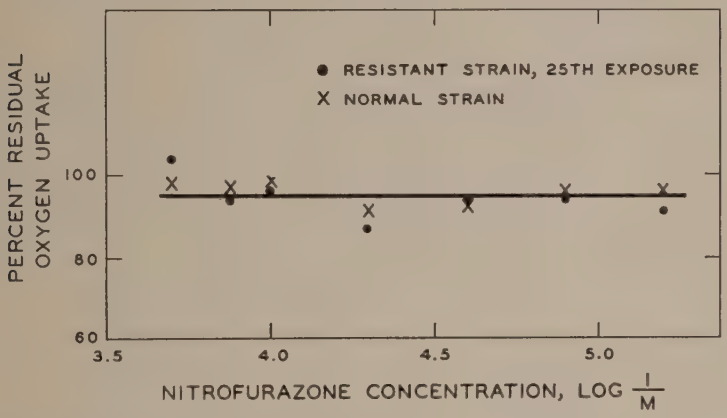

Fig. 6 Influence of nitrofurazone on the oxygen consumption of normal and arsenic-resistant *Trypanosoma gambiense*.

of its poor solubility in saline could be used only at high dilutions; it was entirely ineffective against the trypanosomes (fig. 6). The two other compounds are much more water soluble and could therefore be employed at much higher concentrations. These were quite effective in inhibiting the oxygen consumption of the trypanosomes (figs. 7 and 8) and, interestingly enough, the arsenic -resistant strain was definitely and significantly more sensitive to both compounds. This hypersensitivity of the arsenic-resistant trypanosomes was much more pronounced than in the case of cyanide where a situation similar in principle was found. Unfortunately the mode of action of nitrofurans is not known well enough at present to allow a de-

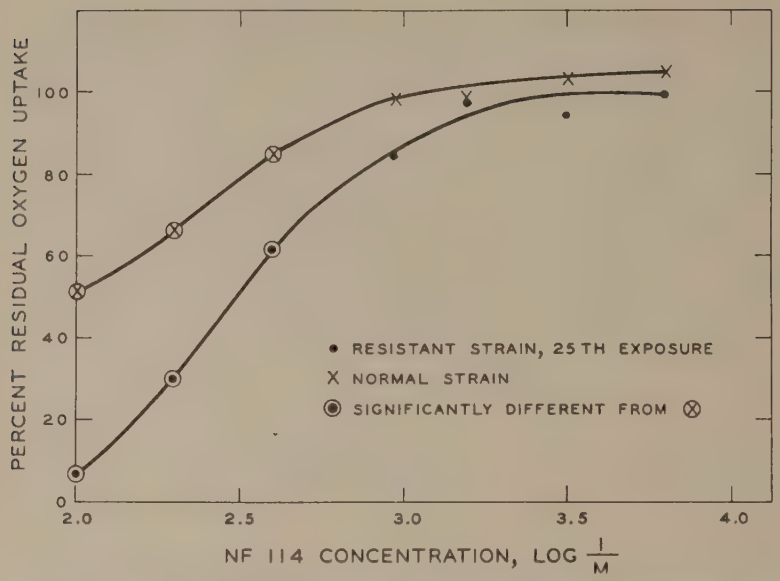

Fig. 7 Influence of NF 114 (5-nitro-2-furaldehyde trimethyl-ammonium acethydrazone chloride) on the oxygen consumption of normal and arsenic-resistant *Trypanosoma gambiense.*

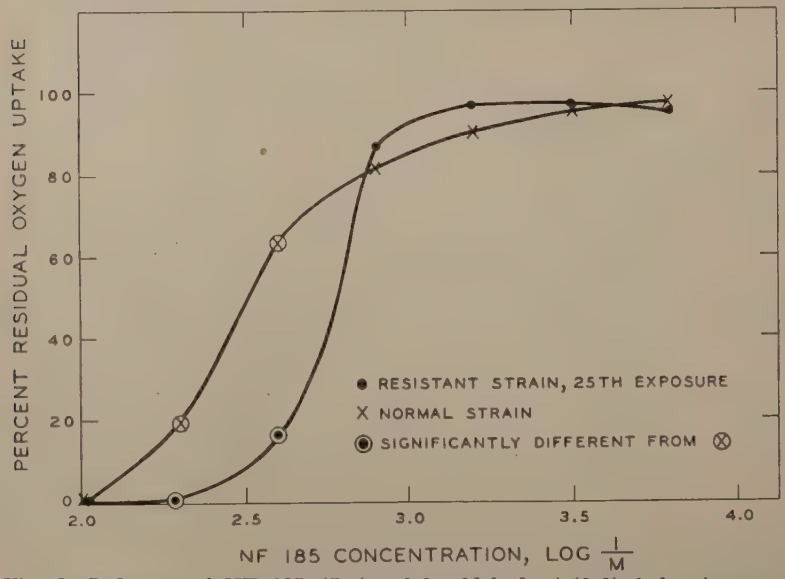

Fig. 8 Influence of NF 185 (5-nitro-2-furaldehyde 4-(3-diethyl-aminopropyl) semicarbazone hydrochloride) on the oxygen consumption of normal and arsenic-resistant *Trypanosoma gambiense.*

cision as to what system or systems were affected in trypanosomes; in other organisms apparently various metabolic systems may be inhibited (Green, '48; Asnis and Gots, '51).

DISCUSSION

The data presented in the preceding sections show that the glucose metabolism of the bloodstream form of *Trypanosoma gambiense* is fundamentally similar to that characteristic of *Trypanosoma equiperdum, Trypanosoma evansi,* and *Trypanosoma hippicum,* where Reiner, Smythe and Pedlow ('36), Marshall ('48), and Harvey ('49) respectively found that glucose is almost quantitatively degraded to pyruvic acid. This result was somewhat unexpected since, according to Fulton and Stevens ('45), the metabolism of *Trypanosoma rhodesiense* is characterized by the predominance of succinic acid formation. *T. rhodesiense* is much more closely related to *T. gambiense* from a taxonomic standpoint than the other species mentioned above. There is a possibility that the differences between the two may be related to different techniques used. We used 40-minute incubation periods at 37°C., while Fulton and Stevens ('45) incubated their flagellates for 6 hours in blood-free solutions at room temperature. It must be expected that in nature within a *Glossina,* the transition from bloodstream form to the developmental form proceeds quite rapidly, at least insofar as the metabolism is concerned. This must be postulated, because the bloodstream form is absolutely dependent on an adequate extraneous source of sugar. It is difficult to see, however, by what mechanism sufficient glucose could be retained in the alimentary tract of the fly for a protracted period, since glucose universally is very rapidly absorbed from the intestinal tract into the tissues. It may therefore be worthwhile to consider the possibility that Fulton and Stevens ('45) were dealing largely with metabolic endproducts characteristic for developmental stages, but obviously only a reinvestigation of *T. rhodesiense* will elucidate this question.

Our strain of *T. gambiense,* rendered about 600 times more resistant to reduced tryparsamide than the original strain,

showed some differences and similarities to the parent strain, which are summarized in table 3.

Our finding that the over-all metabolic rate of sugar consumption and pyruvate production of the resistant strain was slightly lower than that of the parent strain is in contrast to the finding of Harvey ('49) who did not find a similar differ-

TABLE 3

Summary of reactions of an arsenic-resistant strain of Trypanosoma gambiense

PROCESS TESTED	REACTION OF RESISTANT STRAIN, 25TH EXPOSURE AS COMPARED TO PARENT STRAIN
Rate of sugar consumption	Slightly lowered
Rate of pyruvate production	Slightly lowered
Rate of oxygen consumption	Essentially unchanged
Reaction to reduced tryparsamide	600-fold increase in resistance
Reaction to mapharsen	30-fold increase in resistance
Reaction to sodium arsenite	Very slight increase in resistance
Reaction to D,L-glyceraldehyde	Slight increase in resistance
Reaction to iodoacetate, iodoacetamide, fluoride, cinnamate, azide, nitrofurazone, malonate, fluoroacetate	Essentially unchanged
Reaction to cyanide	Very slight increase in sensitivity probable
Reaction to NF 114[1] and NF 185[2]	Definite increase in sensitivity

[1] 5-nitro-2-furaldehyde trimethyl-ammonium acethydrazone chloride.
[2] 5-nitro-2-furaldehyde 4-(3-diethylaminopropyl) semicarbazone hydrochloride.

ence between a normal strain of *T. hippicum* and a strain made resistant to oxophenarsine. However, the latter strain showed only a ten-fold increase in resistance and is hence hardly comparable to our strain.

Our data indicate, and in this point we are in agreement with Harvey ('49), that arsenic resistance does not induce a fundamental change in metabolism. The resistant strain, just

as the normal one, depends on sugar consumption, and the sugar degradation does cease essentially at the pyruvate stage. The lack of reaction to such inhibitors as malonate and fluoroacetate indicates that arsenic resistance is not accompanied by the establishment of a tricarboxylic acid cycle which is completely lacking in the parent strain.

Our data also seem to confirm the view expressed by Yorke, Murgatroyd, and Hawking ('31) and Hawking ('37) that arsenic resistance is not due to an increase in the sulfhydryl content of the trypanosomes which would increase their detoxicating powers (Voegtlin, Dyer and Miller, '24). This is very clearly indicated by a complete lack of resistance to such sulfhydryl inhibitors as iodoacetamide and iodoacetate.

Yorke and Murgatroyd ('30) found, in biological tests, that trypanosomes made resistant to organic arsenicals showed no resistance whatever to sodium arsenite, and they are inclined to assume that a so-called arsenic resistance is no true resistance to arsenic, but a resistance to the substituted phenyl radicals. While we, too, found a very pronounced discrepancy between arsenite and organic arsenicals and hence hold the above view to be essentially correct, our more delicate testing technique has indicated that resistance to reduced tryparsamide is accompanied by a very slight increase in resistance to arsenic proper.

We are also in essential agreement with the British workers in assuming that the mechanism of arsenic resistance consists largely in a change in permeability. The enormous difference in reaction to reduced tryparsamide and arsenite would otherwise be difficult to understand. On the other hand, however, we believe our data indicate rather clearly that permeability changes alone are hardly sufficient to explain fully all our observations. The following evidence points in this direction. The slight increase in resistance to arsenite is accompanied by a similar increase in resistance to D,L-glyceraldehyde; both compounds probably interfere with hexokinase. We found furthermore that a slight increase in sensitivity to cyanide was probable and that a very definite increase in sensitivity to

nitrofurans occurred. This, incidentally, appears to be the first instance of observed increased sensitivity to any compound in drug-resistant trypanosomes, an observation which might be of some importance in drug screening programs. Obviously, on paper at least, it is possible to postulate that the decrease in permeability to arsenicals and glyceraldehyde was accompanied by an increase in permeability to the nitrofurans and to explain in this way practically any change that may become apparent between a normal and a drug-resistant strain. We are more inclined to assume that a certain change in intermediate metabolism has occurred. While we do not think that our data indicate that a completely new pathway has become established, we do think it entirely possible that an existing alternate pathway plays a more important role in the resistant trypanosome than in the normal one. A definite decision will probably require the application of new techniques, to study their intermediary metabolism in more detail.

SUMMARY

1. The bloodstream form of *Trypanosoma gambiense* degrades glucose to pyruvic acid; the molar ratio between glucose consumed, pyruvate produced, and oxygen consumed is very close to the ratio 1:2:1, indicating that practically all the oxygen consumed is used in the above sequence.

2. A strain of *T. gambiense* rendered 600 times more resistant to reduced tryparsamide than the original strain, consumed somewhat less glucose and produced somewhat less pyruvate than the parent strain.

3. The tryparsamide-resistant strain, showed a considerable resistance to mapharsen, and a much slighter one to sodium arsenite and D,L-glyceraldehyde.

4. The arsenic-resistant strain showed a suggestion of increased sensitivity to cyanide and a definite increase in sensitivity to two nitrofurans.

5. The arsenic-resistant strain showed unchanged reactions to 8 other inhibitors.

6. It is concluded that the mechanism of arsenic resistance is primarily a change in permeability, but that the utilization of an already existing metabolic bypass is also probable.

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ANAEROBIC NUTRITION OF SACCHAROMYCES CEREVISIAE

I. ERGOSTEROL REQUIREMENT FOR GROWTH IN A DEFINED MEDIUM [1]

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Past work in this laboratory has shown that the stationary populations of *S. cerevisiae* produced in a yeast extract medium during serial transfer under continuous anaerobic conditions were quite small, while excellent growth occurred in the presence of oxygen (Brockmann and Stier, '47). It was also shown that the anaerobic population could be greatly increased by supplementing the medium with various crude materials, such as vegetable oils, malt sprouts and distillers' dried solubles, or their unsaponifiable fractions. This result indicated that sterols might have special significance in the anaerobic growth of yeast (Stier and Scalf, '49; Stier et al., '50; Scalf and Stier, '50).

In order to proceed with studies on the role of sterols in anaerobic growth, it was necessary to change from the yeast extract medium that had been used in previous work to a chemically defined basal medium. It is the object of this report to describe this medium and especially to show the effect on anaerobic growth of adding a yeast sterol, ergosterol, to this medium. A comparison of anaerobic and aerobic growth is also given.

EXPERIMENTAL PROCEDURES

Yeast strain. All experiments were conducted with a distillery type yeast; a strain of *S. cerevisiae,* SC-1 (DCL),

[1] This work was aided by a grant from Joseph E. Seagram & Sons, Inc.

[2] Seagram Research Associate at the Laboratory of Cell Physiology, Indiana University, assigned by Joseph E. Seagram & Sons, Inc., Louisville, Kentucky, February, 1950.

obtained from Joseph E. Seagram and Sons, Inc., Louisville, Kentucky.

Growth criterion. The number of yeast cells in a known dilution of culture was determined by direct count in a Neubauer chamber. All buds the size of the smallest independent cell were included in the count.

Removal of oxygen. Anaerobic conditions were obtained by thoroughly flushing all experimental vessels and media with Linde "high-purity dry nitrogen." The details of its use are described below. The manufacturer claims 99.99% purity, the impurities consisting of oxygen, carbon dioxide and inert gases. The oxygen content is claimed to be less than 20 p.p.m. In some experiments, the nitrogen was passed through a solution of chromous chloride to reduce the oxygen concentration further (to *ca.* 1 p.p.m.). The chromous chloride was prepared by means of a Jones reductor.

Defined basal medium. The basal medium consisted of Difco Yeast Nitrogen Base, sodium succinate buffer and glucose. The formulation for one liter is given below.

Yeast nitrogen base — 6.7 gm.

a. Ammonium sulfate	5.00 gm		
DL-methionine	0.02 gm		
DL-tryptophane	0.02 gm		
L-histidine	0.01 gm		
b. Salts:			
Potassium phosphate monobasic	1.0 gm	Sodium chloride	0.1 gm
Magnesium sulfate	0.5 gm	Calcium chloride	0.1 gm
Trace elements:			
Boric acid	500 gamma	Potassium iodide	100 gamma
Copper sulfate	40 gamma	Sodium molybdate	200 gamma
Ferric chloride	200 gamma	Zinc sulfate	400 gamma
Manganese sulfate	400 gamma		
c. Biotin	2 gamma		
Calcium pantothenate	400 gamma	*p*-aminobenzoic acid	200 gamma
Folic acid	2 gamma	Pyridoxine HCl	400 gamma
Inositol	2000 gamma	Riboflavin	200 gamma
Niacin	400 gamma	Thiamine HCl	400 gamma

Sodium succinate buffer (0.3 M) (pH 5.0) — 160 ml.
Dextrose (Merck, reagent) — 100 gm.

Difco Yeast Nitrogen Base is manufactured according to a formula originally developed by Wickerham ('51) for use in the classification of yeasts. Its use in the present work should not be construed as an indication that our yeast necessarily requires all of these components. It was selected primarily because of the apparent completeness of its formulation and because of the convenience that it afforded. The basal medium employed is similar to, though more complete in some respects than, those used by other investigators for studies on yeast growth (White and Munns, '51; Winzler et al., '44; Olson and Johnson, '49). The medium also contains a multiple source of nitrogen which Thorne ('50) reports to be beneficial for growth.

A stock solution of Yeast Nitrogen Base was made up aseptically in 10 × concentration (6.7 gm per 100 ml) and stored in the refrigerator. Basal medium for one test flask consisted of 2.5 ml of the 10 × Yeast Nitrogen Base, 4 ml of sodium succinate buffer, and glucose solution. The volume of glucose solution was varied in accordance with changes in volume of inoculum and with the volume of supplements added, so as to give a final volume of 25 ml of medium containing 10% glucose. This concentration was found to be neither limiting nor inhibitory.

Inoculum. Inocula for shake cultures were taken from an anaerobic yeast culture unit (Stier et al., '50) and used in an amount to give initially *ca.* 0.5 million cells/ml (hereafter expressed as M cells/ml). The anaerobic culture unit is an all-glass apparatus that provides a regular supply of inoculum having a continuous anaerobic history. When the culture unit is first put into operation, at least three anaerobic transfers are made before the yeast is used as inoculum for shake cultures.

Operation of anaerobic culture unit. The apparatus described earlier (Stier et al., '50) was scaled down to accommodate smaller volumes, but was otherwise similar in construction and operation except as noted below. The present culture vessel was fabricated from a 90 ml heavy-walled

centrifuge tube and calibrated. A small glass-enclosed Alnico magnet served as the internal portion of a magnetic stirring device. A one-liter flask, covered with metal foil, was used as the medium reservoir.

The anaerobic culture unit was put into operation in the following manner. The entire unit was sterilized and the medium supply flask filled aseptically. The inoculum and the stirring bar (sterilized with ethanol) was put into the graduated culture tube and the unit was then assembled. Nitrogen flushing was started and sufficient medium was transferred to bring the culture volume to 25 ml. The inoculum was taken initially from a yeast extract agar slant and transferred 4 times, aerobically, through the medium which was to be used in the anaerobic culture unit. The anaerobic cultures were transferred every 48 hours in the manner described by Stier et al. ('50). The temperature of the water bath was 26°C. The inoculation rate of *ca* 0.5 M cells/ml of medium was held constant in all experiments reported in this paper.

The nitrogen flow through the unit was normally regulated to give about 20 bubbles per minute through the culture. A greater flow rate seemed to cause sticking of the yeast to the walls of the culture tube. It was found that anaerobic transfers from the culture to shake flasks could be made safely and conveniently by using the transfer flask and hypodermic syringe technique described by Scalf and Stier ('50).

Management of anaerobic shake cultures. All anaerobic shake culture tests were conducted in all-glass equipment consisting of a 250 ml Florence flask equipped with a special head. The head consisted of a male standard taper joint to which was attached a gas trap and a tube for flushing with nitrogen. One end of the tube dipped into the medium and the other end was fitted with a stopcock for sealing the flask after flushing. Although mercury was used in the trap at first, it was found that glycerine effectively excluded oxygen and had obvious advantages over mercury with respect to corrosion and handling.

The basal medium was compounded from stock solutions, as indicated above, and dispensed into sterile culture flasks. Additives were pipetted directly into the flasks, sterile water being substituted in the flasks not receiving additives. The total volume of medium, including inoculum, was 25 ml. After the medium had been placed in the flasks, a hook-shaped wire (20 gauge) was hung on the lip of the flask. The head, with greased joint, was then put in place, but prevented from seating by the wire. The flasks were put on the shaker and flushing with nitrogen started. After flushing for one hour, the inoculum was introduced from a hypodermic syringe by means of a long needle inserted alongside the wire. The joints were then seated and the flushing continued for a half-hour, after which time the stopcocks were closed. The nitrogen flow was regulated from a manifold of needle valves to give a rate of 30–40 cm^3/min. The cultures were incubated in a 26°C. water bath for 48 hours, which time was found adequate to obtain a stationary population. Agitation was provided by a reciprocal shaker operating through a one inch stroke at 110 cycles/minute.

Ergosterol stock solution. A concentrated ergosterol stock solution was prepared by dissolving ergosterol in boiling 95% ethanol and Tween 80 (polyoxyethylene sorbitan monooleate). For example, 50 ml of stock solution was prepared by combining 0.2188 gm of ergosterol, 22 ml of Tween 80 and about 30 ml of ethanol. After boiling, the solution was transferred to a volumetric flask and made to volume with ethanol. When 0.4 ml of this solution was used in 25 ml of medium, the medium contained 7 mg % ergosterol and 0.7 volume per cent Tween 80. For lower concentrations the stock solution was first diluted 1/10 with water. Use of this stock solution results in the presence in the medium of a fractional percentage of ethanol which is partially removed during flushing with the nitrogen gas. It was found that there was no adverse effect on anaerobic growth if even greater concentrations of ethanol were added initially.

Aerobic conditions. Aerobic conditions were maintained in the shake flasks by playing a stream of moist, sterile air (30 cm^3/min.) on the surface of the culture fluid.

EXPERIMENTAL RESULTS

Exploratory experiments in shake flasks

Anaerobic growth in the defined basal medium. A yeast extract medium had been employed in previous growth studies in this laboratory and it was found that 5–7% Difco yeast extract was required to attain a maximum stationary population of *ca.* 100 M cells/ml (Scalf and Stier, '50); the concentration giving half-maximal populations was 1.7%. It was also found that a number of different lots of Difco yeast extract had only about one-fifth of this activity. For these reasons, and because of the complex nature of yeast extract, it was obvious that a defined basal medium was required for a study of anaerobic growth requirements.

Yeast Nitrogen Base, buffer and dextrose, in the concentrations mentioned above, were selected as the basal medium ingredients. Heavy growth of 400 M cells/ml was obtained in this basal medium by aerobic serial transfer. This was taken as evidence that the medium was adequate under conditions where oxygen was present.

Under anaerobic conditions, however, the basal medium produced only one or two generations. When inocula grown in the anaerobic culture unit on a 0.7% yeast extract medium were used, stationary populations of only 1–2 M cells/ml were obtained in shake flasks. This is in accord with the finding of White and Munns ('51). For these exploratory experiments the inocula were washed three times with freshly autoclaved distilled water and the cells resuspended after centrifuging by bubbling with nitrogen. Even this low value may be somewhat high in view of possible oxygen contamination by this washing procedure (see experiments using the anaerobic culture unit reported below). It was apparent, therefore, that the basal medium was adequate for aerobic

growth, but that some factor(s) for anaerobic growth was lacking.

A number of non-lipide compounds was incorporated into the basal medium in an effort to increase the yield of cells under anaerobic conditions. These included yeast nucleic acid, purines, pyrimidines, amines, simple polypeptides, and vitamin B_{12}, all of which were ineffective. Acid and enzymatic casein hydrolysates were likewise ineffective. Since attempts to stimulate anaerobic growth in the basal medium by various known non-lipide compounds were unsuccessful, the lipide phase of the work was then undertaken.

Anaerobic growth in a defined basal-ergosterol medium. A sterol was chosen because, as mentioned previously, the unsaponifiable fractions of various crude materials were found to increase the anaerobic stationary populations (Stier et al., '50). Ergosterol was selected because it is a normal constituent of yeast and is available in relatively pure form. Two commercial sources, Eastman and Light (England), were tested and found to be equivalent. The Eastman ergosterol was used routinely. Since ergosterol is insoluble in water it was necessary to employ a surfactant to render it soluble. Preliminary tests revealed that the full impact of ergosterol on anaerobic growth could not be realized except by very fine emulsification. To eliminate variations that might occur because of inadequate emulsification, the solubilization technique, described in the procedure section, was devised so that ergosterol could be put into apparent solution.

Exploratory shake flask experiments were performed with ergosterol to determine its impact on growth in the defined medium and to determine the optimal concentration. Washed 0.7% yeast extract inocula from the anaerobic culture unit were again used for these experiments. The ergosterol concentration was varied from 0.05 to 7.0 mg %. Approximately 0.2 mg % was found to be optimal, giving a stationary population of 40 M cells/ml. Identical populations were obtained at 7 different concentrations above the optimum. The effect of Tween 80 alone was determined in the

anaerobic culture unit and will be described in the following section.

Experiments in the anaerobic culture unit

The above exploratory experiments were conducted using a washed inoculum grown anaerobically in a yeast extract medium and therefore do not indicate the populations that would obtain under continuous anaerobic subculture in a defined medium. This result can best be obtained by use of the anaerobic culture unit. In addition, continuous anaerobic cultures grown in a defined medium were required as inocula for shake flask experimentation. Such a culture procedure obviated the necessity for washing the inoculum between transfers and so eliminated the possibility of oxygen exposure.

Three media were tested for their capacity to support growth during subculture in the anaerobic culture unit; these media were (a) basal, (b) basal-Tween 80 and (c) basal-Tween 80-ergosterol. The initial inoculum for each series was prepared as described in the procedure section.

Due to the presence of some oxygen in the culture unit initially, growth was obtained in the basal medium for the first two transfers, but no growth occurred in the third subculture. Growth in the basal-Tween 80 medium decreased in the same manner with the same result, provided that the flushing nitrogen was first passed through a chromous chloride tower to reduce the oxygen content of the gas. Without this absorption, the population would frequently stabilize at 3–5 M cells/ml. The Tween 80 concentration used was 0.7 volume %, the amount used in the ergosterol experiments reported below. Preliminary experiments had shown that Tween 80 used over a wide concentration range was not inhibitory under anaerobic conditions, as tested in combination with ergosterol.

Although neither the basal nor the Tween 80-basal media would support growth in the anaerobic culture unit, the solubilized ergosterol medium containing 7 mg % of ergosterol was again found to promote considerable proliferation. One

unit has been in continuous operation for over 5 months and has maintained an average population of 116 M cells/ml during this time. Three other units were operated over shorter intervals and maintained average populations of 109, 122 and 124 M cells/ml. It should be mentioned here that contamination has not been encountered. When yeast from the culture unit was employed to inoculate a similar ergosterol medium in shake flasks, the anaerobic populations were the same as those obtained in the culture unit.

The optimal ergosterol concentration was redetermined, this time using anaerobic shake cultures inoculated with yeast grown in a 7 mg % ergosterol medium in the anaerobic culture unit. The volume of inoculum was 0.1 ml so that the ergosterol added in this way was not great enough to materially affect the initial concentrations in the test flasks. The optimal concentration was found to be approximately 0.7 mg % under these conditions. The populations obtained in the range of 0.7 to 7.0 mg % were of the same order of magnitude, indicating that ergosterol had no inhibitory effect over this range. The seemingly high ergosterol concentration of 7 mg % was employed in the culture unit to supply inocula for experiments to be reported later.

The ergosterol subcultures, unlike the Tween 80 cultures, did not exhibit a sensitivity to small traces of oxygen. When chromous chloride was employed to reduce the oxygen content of the nitrogen used in flushing, the average population was the same as when the absorbent was not employed.

The populations obtained in the culture unit are dependent upon the history of the inoculum employed in starting the unit. When the starting inoculum was derived from aerobic subcultures made in basal medium without ergosterol, the populations in the anaerobic culture unit averaged 70 M cells/ml (for transfers no. 4–13) and the maximum value was not reached until after several weeks of subculturing. This sequence of changes in the magnitude of the stationary populations was not obtained when the starting inoculum was derived from yeast subcultured aerobically in the ergo-

sterol medium. By the latter procedure the initial populations were found to be in the maximal range of 110 to 125 M cells/ml.

Aerobic experiments

The effect of oxygen on growth in the three defined media has not been studied in detail as yet. Consequently, definite conclusions cannot be given at this time. We have employed only one aerobic procedure thus far, namely: cotton-plugged shake flasks bearing an inlet tube through which a stream of air was played on the surface of the culture fluid; and washed inocula derived from the anaerobic culture unit operating on the ergosterol medium. It was found that the three defined media gave the following average stationary populations after 4 aerobic subcultures: basal, 400 M; Tween 80-basal, 350 M; and ergosterol-Tween 80-basal, 300 M cells/ml. Thus, excellent growth was obtained without ergosterol, although the data indicate that added Tween 80 and ergosterol were somewhat inhibitory under these aerobic conditions.

DISCUSSION

Examples of the growth promoting action of sterols or steroids under anaerobic as well as aerobic conditions have been reported for various organisms. Some species of parasitic trichomonads required cholesterol, or chemically similar sterols, for reproduction. These flagellates grow best under anaerobic conditions (Lwoff, '51). Prickett and Massengale ('31) found that strains of Mycobacterium contained no ergosterol and that this sterol stimulated growth. Devloo ('38) isolated a sterol, biosterol, which he found to be indispensable for Wildier's yeast. Ergosterol, sitosterol and calciferol were equally active, presumably under an aerobic state achieved in cotton-plugged culture tubes. Williams et al. ('49) showed the steroid antistiffness factor to be effective for *L. bulgaricus*. Some flies, beetles and snails have been reported to require cholesterol or plant sterols for growth (Heilbrunn, '43).

An interesting facet of the problem is indicated in studies of sterol synthesis. There is general agreement that sterol synthesis is a feature of aerobic metabolism (Maguigan and Walker, '40; Massengale et al., '32; Prickett et al., '30). Block ('46) has shown that no cholesterol is produced by liver slices anaerobically. Some bacteria appear to be devoid of sterols, for example, Mycobacterium (Prickett and Massengale, '31) and Corynebacterium and *E. coli* (von Behring, '30), while others such as Azotobacter (Greaves, '35), *B. butyricus* and *Staph. albus* contain appreciable amounts of sterols, as reported by Bills ('35). Various yeast strains contain different amounts of ergosterol (Bills et al., '30). One can speculate that ergosterol is not essential for our strain of yeast aerobically because it is synthesized by the cell but is required anaerobically because of a lack of synthesis. We have not as yet studied these metabolic aspects of anaerobic yeast growth.

That the sterol requirement of the yeast is not confined to ergosterol was shown by exploratory experiments in which other sterols were employed. Cholesterol (Eimer and Amend, C.P.) promoted anaerobic growth, though to a lesser extent than ergosterol. Concentrations above the optimal became increasingly inhibitory, possibly indicating the presence of an impurity. The antistiffness factor (van Wagtendonk and Wulzen, '50) was also tested and found equivalent to ergosterol at the concentration employed. Calciferol and cholic acid were found to be inactive. No attempt has been made as yet to determine the essential molecular configuration.

Although stationary populations of 110–125 M cells/ml have been obtained by serial transfer under anaerobic conditions by the incorporation of ergosterol into the defined basal medium, this value falls short of the large population (300 M cells/ml) attained by the addition of the unsaponifiable fraction of wheat germ oil to a crude medium containing yeast extract (Stier et al., '50). One may assume from this that it should be possible to attain much greater anaerobic populations than have been obtained thus far in the defined ergo-

sterol medium by increasing the concentration of certain of its components and by the addition of anaerobic nutritional factors to be found in crude materials. Work on this aspect of the anaerobic nutrition of yeast is now in progress.

We wish to thank Dr. W. J. van Wagtendonk for a sample of antistiffness factor.

SUMMARY

Chemically defined media were employed for studies on the anaerobic proliferation of *Saccharomyces cerevisiae* and for aerobic comparisons. Special anaerobic techniques and apparatus for continuous anaerobic subculture are described.

It was found that the basal medium was not capable of supporting growth, even though it was supplemented with a variety of non-lipide substances. When ergosterol, solubilized with Tween 80, was added to the basal medium, populations of 110–125 million cells/ml were obtained in continuous subculture; in one case for a period of over 5 months' duration. Tween 80 alone did not support growth in the basal medium. The optimal ergosterol concentration was found to be 0.7 mg % for the anaerobic population range given above. Experimental data obtained thus far indicate that ergosterol is unnecessary aerobically and may be somewhat inhibitory. It is concluded that ergosterol (and certain other sterols and steroids) should be considered as an anaerobic nutritional requirement of the yeast employed in these studies.[3]

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[3] Since submitting this manuscript for publication it has been found that unsaturated C_{18} fatty acids are also essential for anaerobic growth. This requirement is in addition to the sterol requirement; both must be present. In the experiments reported in this paper, oleic acid was supplied by Tween 80. Fatty acid requirements will be considered in a future publication.

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THE ACTION OF TANNIC ACID ON MODEL SYSTEMS FORMED OF CONSTITUENTS OF THE ERYTHROCYTE MEMBRANE [1]

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ONE FIGURE

Previous investigations (Jacobs, Stewart and Butler, '43) have shown that tannic acid (TA) in very low concentrations greatly reduces the characteristic permeability of the erythrocyte to anions and at the same time has little or no effect on the permeability to water and non-electrolytes. Further studies by Edelberg ('52) on the adsorption of TA by the red cell membrane indicate that only a small fraction of the surface is normally permeable to ions. The interpretation of these findings in terms of membrane structure requires information as to which chemical constituent or constituents of the membrane are involved in the changes in ionic permeability.

Chemical analyses of erythrocyte ghosts (Parpart and Dziemian, '40; Erickson et al., '37) have indicated that they consist on the average of perhaps 60% protein, with the phospholipids cephalin and lecithin together accounting for an additional 25% and cholesterol, neutral fats and other less well-known substances comprising the remainder.

[1] This study formed part of a dissertation presented to the faculty of the Department of Physiology of the University of Pennsylvania School of Medicine in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

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The literature offers much information on the reaction of TA with protein but little on interaction with the other components. The following experiments on artificial films and cell models, in part original and in part confirming the work of others, have been undertaken to provide lacking information. They may conveniently be discussed in three sections: (1) cholesterol and fatty acids, (2) proteins, and (3) phospholipids.

1. Cholesterol and fatty acids. A very sensitive method for determining interaction between a monomolecular layer of an insoluble substance on a water surface and a solute in the bulk phase is to measure the change in surface pressure of the film upon injection of the solute into the underlying solution (Adam, '38). Using this method, Schulman and Rideal ('37) found that TA has no significant effect on films of cholesterol and fats. These negative results were confirmed for cholesterol and oleic acid monolayers (table 1), the films retaining their original liquid state after the injection of TA. From the lack of interaction between these compounds and TA, there seems to be little basis for considering them to be involved in the TA effect on permeability.

2. Proteins. Schulman and Rideal ('37) observed that a monolayer of gliadin on water adsorbed TA from the underlying solution. In the process the film was converted into a rigid skin which was resistant to the normally dispersive action of sodium oleate. In view of the acidity of the protein in the erythrocyte membrane, the author chose to perform similar experiments with an acidic protein, casein, instead of the basic one, gliadin. The casein, dissolved in dilute NaOH, was spread by dropping from a low height. It was found that the optimum pH for the TA-casein reaction was below 6.5 as compared to 7.2 reported by Cockbain and Schulman ('39a) for gliadin. Furthermore, the "tanned" casein film was observed to solidify reversibly at low pH (6.5), becoming liquid again if the pH were raised to 8. It should be noted that the type of film reaction occurring here, manifested by a tendency toward solidification and a small drop

in surface pressure (table 1) indicates linkage between polar groups with no penetration of the film by the hydrophobic portions of the solute molecule (Schulman and Rideal, '37).

These results may be useful in identifying a possible reaction between TA and a component of the erythrocyte membrane. The pH relationship is similar to that for the effectiveness of TA in diminishing ionic permeability, a result

TABLE 1

Effect of low concentrations of tannic acid upon monolayers of various components of the cell membrane or related substances

SURFACE FILM	pH	FINAL COCENTRATION OF TANNIC ACID	CHANGE IN SURFACE PRESSURE AFTER INJECTION OF TANNIC ACID	FINAL STATE OF THE SURFACE FILM
		%	*dynes/cm*	
Oleic acid	6.5	0.01	No effect	Liquid
Cholesterol	6.5	0.001	No effect	Liquid
Lecithin	6.5	0.0005	+ 4.71	Liquid
Cephalin	6.7	0.001	+ 4.65	Liquid
	6.5	0.0005	+ 3.15	Liquid
Casein	7.6	0.002	— 0.84	Liquid
	7.6	0.004	— 0.93	Viscous liquid
	7.6	0.006	— 0.99	Solid
	6.7	0.002	— 1.08	Very viscous
	6.7	0.006	— 1.41	Solid

to be expected if stromatin with an isoelectric point in the region of pH 5 (Jorpes, '32; Furchgott and Ponder, '40) is involved. Results showing the effect of pH on retardation of ionic movement across the erythrocyte membrane are shown in table 2.

Since TA solidifies a protein film and prevents its dispersal by sodium oleate, it is possible, according to Schulman and Rideal, that it is acting by linking together sev-

eral molecules of protein by virtue of its polymer form. This mechanism seems particularly plausible as an explanation of the protective effect of TA against the hemolytic action of soaps (Buckendahl, '33). It is also at least conceivable that by such a mechanism TA might sterically interfere with the movement of anions across the erythrocyte membrane.

Even more suggestive of the decrease in ionic mobility encountered in the experiments with the erythrocyte is the work of Dean, Curtis and Cole ('40) who observed a large decrease in electrical conductivity when TA was applied to an ingeni-

TABLE 2

Effect of pH on the retardation of hemolysis in M/6 NH_4Cl by tannic acid

pH	TIME FOR 40% HEMOLYSIS		RATIO: time (tannic acid) / time (control)
	Control	0.005% tannic acid	
6.3	7 min. 17 sec.	7 hrs. 20 min.	60.2
7.2	5 min. 6 sec.	2 hrs. 2 min.	24.0
8.1	3 min. 50 sec.	12 min. 5 sec.	3.3

ously formed bimolecular protein film separating two aqueous phases. The film, prepared by pushing together in oil two protein-coated aqueous drops, was believed by Dean ('40) to be free of the original intervening oil layer.

This experiment has been repeated and confirmed in this study, the materials used being Nujol, a mineral oil, egg albumin, 0.01 N KCl and approximately 0.005% TA. Conductivity was measured with a 1000 cycle bridge. The results obtained with the rather crude technique employed showed that the resistance increased from approximately 1 ohm cm^2 in the absence of TA to approximately 28 ohm cm^2 in its presence. These figures are very roughly of the same order of magnitude as the values of 0.5 ohm cm^2 and 42 ohm cm^2 respectively, reported by Dean ('39) and by Dean, Curtis and Cole ('40).

A point that requires particular emphasis is that the mere combination of TA with protein is not in itself sufficient to produce a noticeable reduction in permeability to ions. The molecular organization of the protein structure proves to be of great importance. For example, the electrical conductivity of a 30% gelatin gel containing 0.1 N NaCl was found to be practically unaffected after a long exposure to 0.005%, 0.1% or 1% TA. This was the case whether the gelatin membrane

TABLE 3

Effect of tannic acid on the electrical resistance of 30% gelatin gels containing 0.1 N NaCl

	GELATIN DIAPHRAGM IN TUBE 6 CM LONG		THIN LAYER (1 MM) OF GELATIN POURED ON A POROUS COLLODION MEMBRANE (2.5 CM^2)	
Resistance before exposure (ohms)	8950	7960	81.9	77.2
Resistance after exposure to 0.1% tannic acid (pH 3.5)	....	8250	...	80.7
Resistance after exposure to HCl (pH 3.5)	9240	....	83.9	...
Increase in resistance (ohms)	290	290	2.0	3.5

was in the form of a U-tube, approximately 6 cm long, similar to those originated by Matsuo ('23), or was deposited in a thin layer on a supporting disc of porous collodion (table 3). In another instance, following the work of Loeb ('19, '20), collodion membranes of the megapermselective type developed by Carr and Sollner ('44) were exposed to dilute solutions of egg albumin, casein or gelatin for 24 to 48 hours at 6°C., pH 5.3. In no case was the conductivity of these treated membranes significantly altered by TA treatment, although they had adsorbed protein, as was indicated by the change in concentration potential with pH (table 4).

3. Phospholipids. While observations of the action of TA on isolated proteins appear to furnish a reasonable basis for interpreting its effects on the erythrocyte, it is impossible from the data to be found in the literature to exclude the phospholipids as the site of a similar and perhaps more pronounced effect upon the permeability to ions. On the basis of the findings of Cockbain and Schulman ('39b) that monolayers of long-chain amines react with TA, it was thought that phospholipids by virtue of their basic nitrogen groups might behave similarly. The remaining experiments are concerned with answering this question.

TABLE 4

Effect of tannic acid and of pH change upon the characteristics of protein-coated porous collodion membranes. CoP refers to the concentration potential, 0.1 N KCl/membrane/0.01 N KCl. The electrolyte used for resistance measurements was 0.1 N NaCl, pH 4. Membranes were sealed over the ends of open cylinders, diameter, 2 cm.

PROTEIN USED	RESISTANCE (OHMS PER MEMBRANE)		pH (Before exposure to tannic acid)	CoP (Before exposure to tannic acid)
	Before exposure to tannic acid	After exposure to tannic acid (0.005%)		
Casein	830	835	7	+11
			4	−3.4
Gelatin	1344	1339	10	+7
			2.5	−26

(a) Surface films on water. As a source of relatively pure unoxidized lecithin and cephalin, phospholipids were extracted from dog-brain by a modification of the method of Bloor ('26). Monomolecular layers of these substances were investigated by the trough method already referred to. After injection of TA under the films, there occurred an immediate rise in surface pressure indicating a weak penetration by TA, the resulting films remaining liquid, however. These results (table 1) cannot be attributed to the surface activity of TA per se, since even at concentrations 50 times greater, TA was found to have only a negligible effect on the surface

tension of pure water. According to Schulman and Rideal ('37), film penetration under these conditions, evidenced by an increase in surface pressure, indicates an interaction between the polar groups of the two compounds and also between their hydrophobic portions. The rise in surface pressure in the case of lecithin was about 50% greater than that for cephalin. It must be stated that both the lecithin and cephalin fractions were partly contaminated by each other. The effect of pH upon the mangitude of the TA interaction with phospholipid parallels the pH effect on the reaction with casein (fig. 1).

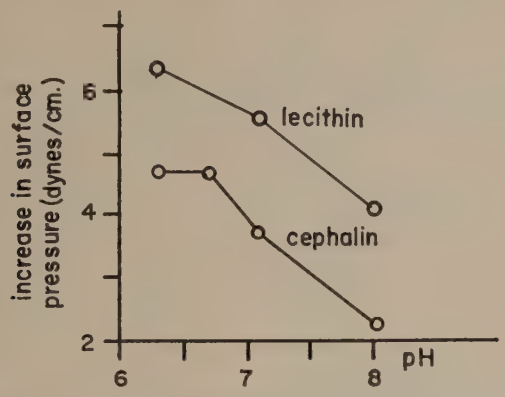

Fig. 1 Effect of pH on the penetration of monomolecular films of phospholipids by tannic acid. The concentration of tannic acid was 0.001%.

On the basis of the reaction between TA and the phospholipids observed in surface films, the specific site of the TA reaction with the cell membrane, whether with the protein or with the phospholipid portion, becomes uncertain.

(b) Lipid membranes. The importance of the reaction of TA with phospholipid as a causative agent in the decrease in the permeability of the erythrocyte could only be evaluated if the effect of TA on the permeability of a phospholipid film were known. To determine this, it was necessary to obtain a pure phospholipid membrane which would be measurably permeable to ions. Stability was also a requisite and although Danielli ('35) had been able to form spherical shell films of

various lipids, these were stable only in the presence of protein. The above requirements were ultimately met by the formation of spherical membranes from a concentrated solution of lecithin in chloroform, this solvent being somewhat water-soluble and hence rather readily displaced from the film by water. A high concentration of lecithin was necessary to insure a packed Gibbs layer because of its high solubility in chloroform.

In a typical set-up, using this method, a layer of saturated NaCl supports a layer of 0.9% NaCl, these aqueous layers locating upon a layer of chloroform containing 0.045 gm of lecithin per milliliter. A dropper, drawn out into a capillary with an orifice 1 mm in diameter, is filled with 0.9% NaCl and inserted into the lipid layer. The droplets of expelled saline will rise into the aqueous phase coated with a thin lipid membrane and will float at the junction of the two NaCl layers. Spheres 1 mm in diameter will endure up to 20 minutes. If oleic acid is added to the lipid mixture (e.g. 0.045 gm lecithin plus 0.07 ml oleic acid per milliliter of $CHCl_3$) resistance to rupture is markedly increased, the spheres often lasting for hours, provided the lipid solution has been freshly prepared.

Spheres thus formed behaved as osmometers, rupturing if transferred to hypotonic solutions, and shrinking in hypertonic surroundings. They also proved to be rather freely permeable to ions, unlike the lecithin-impregnated collodion membranes formed by Weatherby ('49). With brom cresol purple and dilute NaOH inside (pH 7.8), the spheres were observed to change color when the external medium was brought to pH 4.5 with HCl. The possibility that CO_2 could be the cause of this shift was eliminated by the use of freshly boiled water and by other means. Permeability to Fe^{+++} and to SCN^- was also demonstrated. For example, if the sphere contained $FeCl_3$ and the external medium KSCN (or the reverse), the deep red coloration of $Fe(SCN)_3$ pervaded the entire sphere within two minutes.

The simplest quantitative measure of the effect of TA on the permeability of these spheres is the time necessary for the color change of a pH indicator, as described above. Experiments involving either 0.005% or 0.02% TA indicated that there is, if anything, a slight acceleration of the rate of change in the presence of TA (table 5). The presence or absence of oleic acid did not affect the comparative results.

Since color tests for ions other than H^+ and OH^- met with objections in the presence of TA, resort to chemical analysis of the sphere contents as a means of following their penetration was taken. Quantitative studies of the penetration of

TABLE 5

Effect of tannic acid upon the permeability of lecithin spheres to H^+ or OH^-. Values given are the averages of readings on at least two groups of 6 spheres each. Differences in control times are due to differences in average diameters.

EXPERIMENT NUMBER	TIME IN SECONDS FOR COLOR CHANGE		
	Control	In presence of 0.02% tannic acid	In presence of 0.005% tannic acid
1	15.5	16.0	
2	22.0	17.5	
3	117	101	
10	20.7		20.3
11	27.0		22.5
12	44.7		40.1

SCN^- were made. In such tests the dropper to be used contains a layer of the previously described lipid mixture above which is a layer of 22.5% sucrose containing 0.9% NaCl. The sucrose serves as ballast in a subsequent operation. The exposure vessels are a series of vials, each containing a layer of 2.5% KSCN in 18.8% sucrose over a layer of 2.5% KSCN in saturated NaCl. Some vials contain TA in both layers. With the orifice of the dropper placed at the bottom of a vial, the lipid layer is forced out and after it several droplets of the sucrose-NaCl solution to form spheres. All but a small part of the lipid layer is sucked back into the dropper

before it is removed to be wiped clean and inserted into the next vessel for another such cycle.

To carry out the analysis of the amount of SCN^- which has penetrated, it is necessary to wash the spheres free of the external solution without removing any of the contents. To this end they are transferred by dropper to a column of 0.9% NaCl at the bottom of which is a layer of a benzene-chloroform mixture, 3 : 1 by volume. The sucrose-laden spheres sink quickly through the saline and pass into the non-polar solvent layer, leaving behind any solution that may have been carried over with them. When a sufficient numbers of spheres has been collected, the aqueous layer of the column is drawn off, the residual SCN^- rinsed away, and a measured volume of sphere contents (e.g. 0.04 ml) withdrawn. This is mixed with 1 ml of 0.2 M $FeCl_3$ in a flat-bottomed cuvette and a reading taken on the Klett-Summerson colorimeter fitted with a green filter. Tests showed that no contamination by external medium was occurring in the wash process. Examples of the degree of reproducibility of the method are shown below. The reading for a sample which had not been exposed to SCN^- was 155. These readings are of 4 separate runs with a 10 minute exposure:

342 341 331 336

A comparison was made of the rates of entry of SCN^- in the presence of 0.01% or 0.0008% TA or of HCl at the same pH (4.5). The resuts do not indicate any significant difference in permeability to SCN^- in the presence of TA (table 6). These results, together with those involving a shift of H^+ or of OH^- make it fairly certain that the ionic permeability of the artificial lecithin membranes here employed is not diminished by TA. Stable cephalin membranes have thus far not been produced for testing.

One circumstance which casts doubt on the significance of the results with lecithin spheres is the fact that such spheres when presumably coated with egg albumin were not affected by TA. This result is surprising in view of the contrary ones with bimolecular films of egg albumin. The explanation for

this discrepancy may be that a particular molecular configuration of the protein is necessary if TA is to affect its permeability, as indicated in the gelatin and collodion membrane studies. Possibly the presence of lecithin in the film produces a configuration not affected by TA.

Of note in the course of attempts to develop a usable lipid membrane was the finding that TA in high concentrations (i.e. 0.5%) would stabilize for hours the protein-free lecithin membranes formed from a benzene solvent. In the absence of TA, these spheres ruptured in 30 seconds. Furthermore, 1% TA prevented the development of myelin forms from a

TABLE 6

Effect of tannic acid upon the permeability of lecithin-oleic acid spheres to SCN^- at pH 4.5. The larger colorimeter readings for a given volume indicate greater penetration. The volumes were different for each experiment.

EXPERIMENT NUMBER	TIME ALLOWED FOR PENETRATION	COLORIMETER READINGS FOR CONTROL	COLORIMETER READINGS FOR SAMPLES RUN IN 0.01% TANNIC ACID	COLORIMETER READINGS FOR SAMPLES RUN IN 0.0008% TANNIC ACID
I	0	153	153	
	5 min.	166	187	
	10 min.	195	195	
II	10 min.	342		347
III	10 min.	318		312

dab of lecithin covered with dilute HCl. Since these effects are observable only at concentrations hundreds and even thousands of times higher than those used for obtaining the decrease in the permeability of the erythrocyte, their relation to the problem of the permeability effect does not seem very great.

Admittedly phospholipid in the cell membrane may be associated with protein (Chargaff and Ziff, '39) and perhaps with other agents (Folch, '42). This could alter the picture. However, on the basis of what has been observed of the TA action on lecithin and lecithin-oleic acid films, it seems unlikely that the effect of TA upon the permeability of the erythrocyte is to be explained in terms of a reaction with phospholipid. On

the other hand it is rather conceivable that the decrease in osmotic resistance of the erythrocyte produced by very low concentrations of TA (Bohlmann, '44; Edelberg, '52) may be ascribed to penetration of the phospholipid portion of the membrane by TA as occurred in the studies on monolayers.

DISCUSSION

Of the various known components of the erythrocyte membrane, TA has been demonstrated to react only with protein or phospholipid. The results of the experiments involving these two groups of substances have been interpreted as supporting the idea that TA imposes its effect on permeability by virtue of a reaction with a protein structure. At the same time they bring to light the possibility that phospholipids *may* be involved in the effect although thus far indications are that they are not.

If the site of action of TA is truly a protein structure, certain deductions regarding the mechanism of the effect appear reasonable. In the case of chemosorption such as probably occurs in the reaction of TA with the cell (Edelberg, '52), the polar regions of the TA molecule, i.e. the phenolic groups, are thought to combine with the keto-imido groups of the protein (Kruyt, '22; De Jong, '23), causing the hydrophobic portion to be directed toward the aqueous phase and the reacting area of the membrane to become hydrophobic. This, according to Schulman and Rideal, is the explanation for the agglutinating action of TA on the erythrocyte. The now hydrophobic surface could be thought to resemble a non-polar oil in the high resistance it offers to the passage of ions.

The situation is probably not quite as simple as this, however. Astbury ('40) found by x-ray diffraction that the side-chain spacing of collagen is increased by TA but at the same time, unlike that of the untreated sample, becomes unalterable by wetting. From Astbury's findings, it appears likely that a hydrophobic structure, presumably the benzene ring of the TA molecule, is inserting itself in the side-chain spaces of the protein molecule. If TA acts on the cell membrane simi-

larly and thus blocks ionic movement, it would imply that protein guards the gateway to the inside of the cell insofar as ions are concerned. This does not preclude the existence of a phospholipid layer below the outer protein.

Inasmuch as the movements of water and small fat-insoluble molecules, in contrast to those of ions, are not impeded by low concentrations of TA, it seems possible that these substances may enter the erythrocyte by a different route, either entirely or alternatively. However it is possible that water and small water-soluble molecules pass through the barrier because of their lack of charge or because of their chemical affinities. The last possibility is supported by the observation by Sebba and Rideal ('41) that a tanned protein monolayer does not impede the evaporation of water.

The demonstration of the high degree of permeability of a pure lecithin membrane to ions and water may necessitate changes in some of the models of the erythrocyte membrane now postulated since many are based on the supposition that a lipid area is impermeable to these. If only a small fraction of the membrane is truly ion-permeable (Fricke and Curtis, '35; Edelberg, '52), then the large area containing phospholipid (and oleic acid) may require some additional structure for retarding ions.

SUMMARY

1. In order to identify the reaction responsible for the decrease in the permeability of the erythrocyte to ions brought about by tannic acid, studies on artificial films and membrane were made.

2. Tannic acid has been shown to be capable of reacting with molecular films of proteins, lecithin and cephalin but not of oleic acid or cholesterol.

3. The reaction of tannic acid with protein films is in many respects similar to that with the erythrocyte. Both reactions take place at the same low concentrations of tannic acid, both are favored by low pH and both result in a decreased permeability to ions.

4. Experiments on a newly developed lecithin membrane show that its relatively high permeability to ions is not decreased by tannic acid.

5. The experimental data are consistent with the view that ions enter the erythrocyte at loci of a predominantly protein nature.

ACKNOWLEDGMENT

I am deeply indebted to Dr. M. H. Jacobs for valuable advice on all phases of this work and for his very welcome criticism of the manuscript. I also wish to thank Dr. J. F. Danielli and Dr. M. G. Sevag for their advice on certain problems.

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HISTOCHEMICAL STUDIES OF THE INHIBITION OF ESTERASES [1]

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SIX FIGURES

Esterases, in the stricter sense of the word, are enzymes hydrolyzing carboxylic acid esters of mono- and polyhydric alcohols and phenols. There is a large body of biochemical data available to show that esterases differ from each other in respect to substrate preference and to their behavior towards various activators and inhibitors. The literature of the subject is so vast that it cannot be quoted in detail. Suffice to say that according to the generally accepted classification, esterases can be divided into two large classes, the aliesterases and the cholinesterases (Richter and Croft, '42). Aliesterases hydrolyze carboxylic esters of N-free alcohols. They are subdivided into two groups: lipases, the substrates of which are glycerides of long-chained fatty acids, and esterases, the substrates of which are short-chained fatty acid esters of monohydric alcohols and phenols. Cholinesterases, on their part, are also subdivided into two groups: "true" or specific cholinesterases which hydrolyze acetylcholine preferentially, and "pseudo" or nonspecific cholinesterases which attack choline esters other than acetylcholine (Nachmansohn and Rothenberg, '44). Each group of enzymes has its specific inhibitors and/or activators. The inhibition and activation effects are just as characteristic as the substrate preferences.

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It should be mentioned, however, that the dividing lines between the groups, in fact, even between the two main classes, are somewhat blurred in that many instances of overlapping substrate specificities and activation and inhibition effects are known. For details, the interested reader is referred to textbooks of enzymology (Sumner and Somers, '47; Sumner and Myrbäck, '51).

The histochemical approach offers certain important advantages over biochemical methods in the study of enzymes. Tissue extracts contain a large number of enzymes which can be separated only with considerable difficulty; accurate separation can be accomplished only if the solubility properties of the individual fractions are exactly known. In histochemistry it is possible to observe a large number of different enzymatic behaviors simultaneously and under a variety of conditions, without going through laborious separation processes. Furthermore, since enzymatic activity is studied *in situ*, localized high concentrations of enzyme in areas as small as a single cell, surrounded by thousands of inactive cells, can be recognized with ease. In test tube experiments, such activities would be wiped out by the dilution effect.

Only a few histochemical studies on the specificity of esterases have been published. Gomori ('48a) showed that lipase and esterase behave histochemically (by the Tween technique) very much like they do in the test tube; they are activated and inhibited by the same substances. He also demonstrated ('49) the unique ability of pancreatic and gastric lipase to hydrolyze unsaturated Tween-like compounds. Nachlas and Seligman ('49) found that the distribution of enzymatic activity as seen with the use of β-naphthyl acetate as a substrate is similar to, but not identical with, that obtained by the Tween method. With the long-chained fatty acid ester method (Gomori, '48b) for cholinesterase, marked species and organ differences in respect to substrate specificity can be observed; some structures hydrolyze lauroyl choline at a higher rate than palmitoyl choline; others behave the opposite way. Koelle ('50) was able to distinguish be-

tween the "true" and "pseudo" type of cholinesterase by using acetyl and butyryl thiocholine as substrates and di-isopropyl-fluorophosphate (DIPFP) as an inhibitor. Barrett and Seligman ('51) found that taurocholate inhibits esterases of all organs except those of the pancreas if indoxyl acetate is used as a substrate.

In the present studies an attempt was made to classify esterases on the basis of both substrate preference and activation and inhibition effects. The methods used were the Tween, long-chained fatty acid choline ester and azo dye techniques.

MATERIAL AND METHODS

For all methods, small pieces of tissue were fixed in chilled acetone or in a mixture of equal parts of acetone and absolute alcohol, dehydrated in absolute alcohol, carried through alcohol-ether and a 2 to 3% solution of collodion in the ice box, two changes of chloroform at room temperature and embedded in paraffin. As a rule, 10 to 20 small pieces of different organs were embedded and cut in a single block. The following species were included in this study: man, dog, cat, guinea pig, rabbit, rat and mouse. Only the last three were studied in detail.

The Tween and cholinesterase methods were used as described in the original papers (Gomori, '45, '48b).

The azo dye technique of Nachlas and Seligman ('49) had to be modified because it was found impossible to get sharp pictures with 6-naphthyl acetate as a substrate. Under higher powers the pictures invariably appeared blurred, and acidophilic structures in the vicinity of centers of high activity often were intensely stained (Gomori, '50). Apparently, either the coupling of β-naphthol is not fast enough or the azo dye formed does not precipitate immediately but remains in a colloidal solution long enough to permit the nonspecific staining of various structures. It was found that if α-naphthol is substituted for the β-isomer and the pH of the substrate mixture is raised to about 8 the sharpness of the reaction is

much improved. Although in test tube experiments the optimal pH of hydrolysis is around 6.5, azo coupling appears to occur much more promptly at pH 8. The reduced rate of the reaction at this pH is of a distinct advantage. Even at this nonoptimal pH, the evolution of naphthol at sites of high activity may be so rapid that the diazo reagent is used up faster than it can be replenished by diffusion from the neighborhood. Under such conditions, the unbound naphthol will have to travel a certain distance before it meets a fresh supply of the reagent. To avoid diffusion artifacts around centers of high activity it may be advisable to cool the substrate mixture with ice or, even better, to stir it vigorously during incubation. A long, narrow ($\pm \frac{1}{2}$ in.) strip of stainless steel, attached to the shaft of a medium speed electric or air pressure stirrer, and immersed almost to the bottom of the Coplin jar, was found to be a cheap and efficient stirring device. Only two slides can be stained at one time, one in the first and one in the 5th slot of the jar, with the tissues facing the paddle. It should be remarked that with β-naphthol acetate as a substrate it is impossible to obtain satisfactory results even with the maneuvers mentioned.

Another interesting substrate used in these studies was the acetate of naphthol AS (2-hydroxy-3-naphthoic acid anilide). It was prepared by esterifying naphthol AS (obtainable from aniline dye manufacturers) with acetic anhydride in pyridine. It forms white crystals, M.P. 159 $\pm$ 0.5°C. It is very poorly soluble in water, and it is necessary to add some propylene glycol to the substrate mixture to assure a high enough concentration. Naphthol AS acetate is hydrolyzed very much slower than either α- or β-naphthol acetate; azo coupling is very prompt, and the shades of the azo dyes it forms are brilliant.

METHODS

1. The α-naphthol method. Into 50 ml of distilled water blow 0.5 to 1 ml of a 1% solution of α-napthol acetate (Eastman no. 2380) in 50% acetone. Add a few milliliters of a 0.2M solution of Na_2HPO_4 (pH slightly in excess of 8). Add 20 to 50 mg

of Diazo Blue B Salt or Diazo Red RC Salt (DuPont), stir the mixture and filter it into a Coplin jar. Incubate slides at room temperature for 5 to 30 minutes or until the desired intensity of shade is obtained (with Diazo Blue B, purple-black; with Diazo Red RC, rusty red). If necessary, the mixture should be cooled and/or stirred.

2. The naphthol AS method. Into 10 to 15 ml of propylene glycol blow 0.5 to 1 ml of a 1% solution of naphthol AS acetate in acetone; fill up, under constant stirring, with distilled water to 50 ml. Add a few milliliters of 0.2M phosphate buffer of pH $\pm$ 6.5; add 20 to 50 mg of Diazo Garnet GBC Salt (DuPont). Stir mixture, filter it into a Coplin jar. Incubate at room temperature for 30 to 90 minutes or until the desired intensity of shade (carmine red) is obtained.

After incubation, the slides are washed under the tap, counterstained with hematoxylin, differentiated in acid alcohol (1% HCl in 70 to 80% alcohol), washed and mounted in glycerol jelly. In case of α-napthol-Blue B Salt combination, slides may be dehydrated in alcohols and mounted in balsam.

The inhibitors and activators were so chosen as to include the most important substances known to affect the activity of esterases. The substances tested and their conclusions used are as follows:

1. Taurocholate, 0.002 to 0.005M.
2. Arsanilate, 0.001M.
3. Quinine, 0.1M.
4. Fluoride, 0.0002 to 0.002M.
5. Eserine, 10^{-5} and 10^{-4}M.
6. Prostigmine, 10^{-5} and 10^{-4}M.
7. DIPFP, 10^{-6}M.
8. Phemerol (Parke-Davis; a quaternary ammonium base), 0.002M.

The inhibitors were incorporated in the substrate mixture, except for DIPFP. In this case, the slides were preincubated at 37°C. for 30 minutes in a solution of DIPFP, rinsed, and

incubated in the regular substrate mixture to which no inhibitor was added.

For the study of inhibition by fluoride in the Tween technique the method had to be modified slightly because of the insolubility of CaF_2. In preliminary experiments it was found that barium can replace calcium without any important change in the final result, except for the fact that Ba soaps form somewhat coarser crystals than Ca soaps. BaF_2 is sufficiently soluble for a high enough concentration of Ba^{++} and F^- ions in the solution.

OBSERVATIONS

In many tissues the topographic patterns of activity, using α-naphthol or naphthol AS acetate as substrates, are identical (liver, the pancreatic acini, the renal tubules). In other tissues, strikingly different pictures are obtained with the two substrates. In the brain of the cat, for example, there is a sharp localization of activity in the astroglia when naphthol AS acetate is used as a substrate, whereas with α-naphthol acetate there is no activity in the glia, but only some diffuse staining of certain unidentified fiber tracts. In the duodenum of the rat, the α-naphthol method stains mainly the epithelium of the villi while Brunner's glands remain almost unstained; the reverse picture is obtained with the naphthol AS method. A detailed description of the localization of activity as seen with the use of naphtholic substrates will be published separately.

With β-naphthol acetate as a substrate the distribution of activity was in all cases identical with that in the α-naphthol technique. This is rather surprising since naphthol AS is a derivative of β-naphthol.

The benzoates of the two naphthols were also used as substrates. They were hydrolyzed at rates very much lower than the corresponding acetates but the localizations were exactly the same. There is no histochemical evidence for the presence of specific acetyl-esterases (Jansen, Jang and MacDonnell, '47) in animal tissues.

On comparing the results of the various techniques it becomes obvious that some enzymes possess narrowly limited substrate specificities in that they hydrolyze only one type of substrate. These enzymes are (1) true lipase which hydrolyzes Tween-like substrates, the unsaturated ones included; (2) esterase, the substrates of which are naphthol esters, and (3) cholinesterase which hydrolyzes esters of choline. In contrast to these cardinal types, there are other enzymes which are less discriminating and will attack substrates of two or even of all three groups. This topic has been dealt with in some detail by Gomori ('52).

The effect of inhibitors

Only such effects as have not been published previously will be mentioned here. In the Tween and cholinesterase techniques the inhibitors had a rather uniform effect on all tissues. In the azo dye methods, on the other hand, some inhibitors displayed markedly different effects on different tissues.

The hydrolysis of Tween 60 was completely suppressed by fluoride and almost completely by phemerol. With the latter, some activity persisted in liver tissue and in the corpora lutea of the mouse and the rat.

Cholinesterase activity was almost completely abolished by taurocholate, phemerol and by 10^{-5} prostigmine and eserine, especially with lauroyl and myristoyl choline as substrates. The hydrolysis of palmitoyl choline was considerably depressed but not abolished. The effect of quinine on cholinesterase was not studied.

The results of the azo dye methods were not noticeably affected by taurocholate, quinine and phemerol. The effects of prostigmine and eserine were practically nil at the concentration of 10^{-5}M; at 10^{-4}M some inhibition of the activity of the muscle spindles of the mouse was noted. Hydrolysis of naphthol AS acetate by the motor ganglion cells of man, the rabbit and the rat was unaffected.

The effects of fluoride, arsanilate and DIPFP, especially on the results of the azo dye methods, were so varied that

TABLE 1

Inhibition of esterases

Explanation of symbols

0 = no inhibition
1 = moderate inhibition
2 = marked inhibition
3 = almost complete or complete inhibition
X = does not stain in this species
— = not studied

SPECIES	METHOD	LIVER	PAN-CREAS	INTES-TINE	KID-NEY	LUNG AND BRONCHI	NERVE CELLS	TESTIS	MACRO-PHAGES	SPLEEN	MUSCLE SPIN-DLES
					Di-isopropylfluorophosphate						
Mouse	AS	1	3	1	2	2	x	3	x	x	1–2
	α	2–3	2	3	3	3	x	3	x	x	3
	Tween	0	0	0	0	0	x	0	x	x	x
	ChEst [a]	1–2	x	1–2	x	x	2–3	2	x	x	2
Rat	AS	2 [b],0 [c]	2	2	2	3	0–1	2 [d],0 [e]	0	x	x
	α	2–3	2–3	3	2–3	3	x	2–3	x	x	x
	Tween	0	0	0	0	0	x	0	x	x	x
	ChEst	—	—	—	—	—	—	—	—	—	—
Rabbit	AS	1	1–2	1–2	2	2	2	3 [d],0 [e]	3	2–3	x
	α	3	2	3	3	3	x	0	x	0	x
	Tween	0	0	0	0	0	x	0	x	0	x
	ChEst	—	—	—	—	—	—	—	—	—	—
					Fluoride, 0.002 M						
Mouse	AS	2	2	3	3	3	x	3	x	x	3
	α	0	0	0	0	3 [f],1 [g]	x	2 [d],0 [e]	x	x	0
	Tween	3	3	3	3	3	x	3	x	x	x
	ChEst	2	x	1–2	x	x	x	3 [h]	x	x	0
Rat	AS	2 [b],1 [c]	1–2	2–3	3	3	3	3 [d],2 [e]	3	x	x
	α	0	0	0	0	3 [f],1 [g]	x	0	x	x	x
	Tween	3	3	3	3	3	x	3	x	x	x
	ChEst	—	—	—	—	—	—	—	—	—	—
Rabbit	AS	1–2	0	0–1	0	0	0–1	2 [d],0 [e]	0	0	x
	α	0–1	0	0	0	0	x	0	x	0	x
	Tween	3	3	3	3	3	x	3	x	3	x
	ChEst	—	—	—	—	—	—	—	—	—	—
					Arsanilate						
Mouse	AS	3	0–1	3	3	3	x	3	x	x	3
	α	0	0–1	0	0	0	x	0	x	x	0
	ChEst	0	x	0–1 [i]	x	x	2–3	3	x	x	1–2
Rat	AS	2	0	0–1	1	0 [f],2 [g]	0	0	0	x	x
	α	0–1	1	1–2	1	0	x	0	x	x	x
	ChEst	—	—	—	—	—	—	—	—	—	—
Rabbit	AS	3 [b],0 [c]	0–1	3	1–2	0 [f],2 [g]	0–1	3 [d],0–1 [e]	0	1–2	x
	α	0	0	0	0	0	x	x	x	0	x
	ChEst	—	—	—	—	—	—	—	—	—	—

[a] = Effect decreasing with increasing chain length of fatty acid.
[b] = Cells of hepatic cords.
[c] = Kupffer cells.
[d] = Interstitial cells.
[e] = Sertoli cells.
[f] = Septal cells.
[g] = Bronchial epithelium.
[h] = Myristoyl choline.
[i] = Intestinal smooth muscle, 2.

they require tabulation. All effects recorded were those of inhibition; no clearcut instances of activation were observed in these experiments. It should be remarked that histochemical methods do not lend themselves to exact quantitation. Differences amounting to less than ± 50% cannot be recognized with certainty. On the basis of comparison with quantitative Coujard slides (Gomori, '50b), it may be said that grade 1 inhibition in the table corresponds roughly to an inhibition of 50 to 75%; grade 2, to 75 to 90%; grade 3, to 90% or more.

COMMENT

As mentioned, substrate specificities of esterases show a marked tendency to multiple overlapping. The inhibition effects tabulated show an even more irregular and unpredicable pattern than substrate specificities. Enzymes which resemble each other in respect to substrate preference may differ from each other sharply with respect to their response to inhibitors; on the other hand, enzymes with markedly different substrate specificities may be affected in the same way by a number of inhibitors. The behavior of different organs in the same species or of the same organ in different species appears completely erratic. It would be hard to envisage a theory which could explain the bewildering variety of patterns in terms of classical concepts or to fit them into a logical system. It must be assumed that esterases form a large family of closely related enzymes which differ from each other in more or less significant details of one or or several of their prosthetic groups, thus producing the effect of overlapping similarities and divergences.

CONCLUSIONS

Histochemical studies of esterase with various substrates and inhibitors reveal a lack of sharp definition between various types of the enzyme. Substrate preferences and inhibition effects overlap in such an irregular and unpredictable fashion as to defy attempts at classification.

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PLATE

PLATE 1

EXPLANATION OF FIGURES

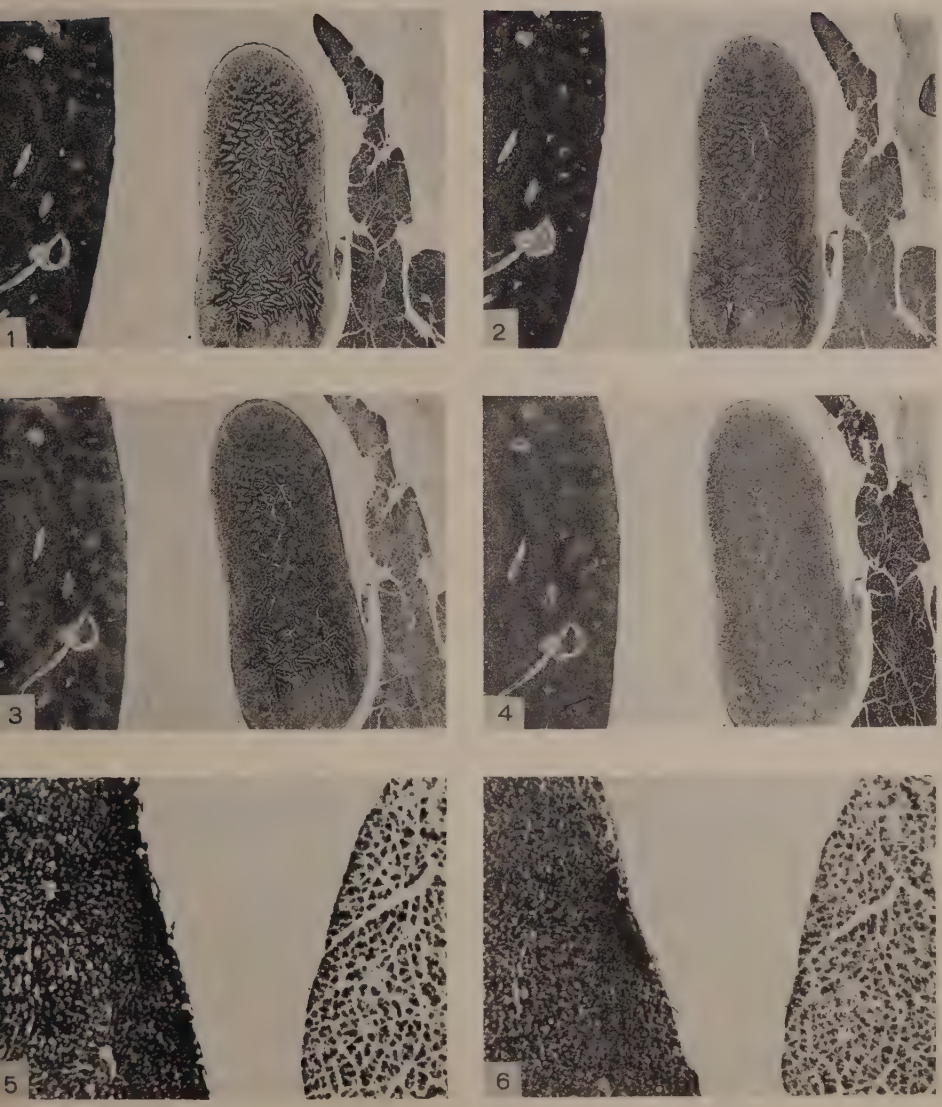
Substrate, naphthol AS acetate; counterstain, hematoxylin.

Figs. 1 to 4 The same area containing pancreas, liver and intestine of consecutive serial sections of mouse organs. × 11.

1 Control section (no inhibitor).
2 DIPFP. Slight inhibition of liver and intestine; almost complete inhibition of pancreas.
3 Fluoride. Marked inhibition of liver and pancreas; complete inhibition of intestine.
4 Arsanilate. Almost complete inhibition of liver and intestine; no inhibition of pancreas.

Figs. 5 and 6 Consecutive serial sections of rat liver and pancreas. × 53.

5 Control section (no inhibitor).
6 DIPFP. Both liver and pancreas markedly inhibited.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THE DECARBOXYLATION OF AMINO ACIDS BY PHOTOBACTERIUM FISCHERI

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FOUR FIGURES

INTRODUCTION

Due to the fact that investigative work in the field of bacterial bioluminescence has been largely directed toward elucidation of the bioluminescent process, it is not surprising to find that there has been little study of metabolic processes not associated with this phenomenon. This study presents part of an investigation of these general metabolic processes, namely the decarboxylation of amino acids by the luminous bacterium *Photobacterium fischeri*.[3] It describes the decarboxylase activities of washed cell suspensions and defines the experimental conditions under which they were determined.

The excellent work on this group of enzymes by Gale and his co-workers which has been reviewed by Gale ('46) has warranted the establishment of certain generalizations in regard to the formation, distribution, and activities of these enzymes. For the most part the results obtained in the present investigation substantiate these generalizations.

[1] Present address: Department of Biochemistry, Vanderbilt University School of Medicine, Nashville 4, Tennessee.

[2] The data in this paper were taken from a portion of a thesis presented by the author in partial fulfillment of the requirements for the degree of Doctor of Philosophy, Vanderbilt University, 1951.

[3] *Achromobacter fischeri* is a synonym for this organism and is used frequently by students of bacterial bioluminescence. Actually this bacterium is listed as *Bacterium phosphorescens indigenus* (Eisenberg) Chester in Bergey's Manual of Determinative Bacteriology ('48); however, this name does not often appear in the literature.

MATERIALS AND METHODS

The strain of *Photobacterium fischeri* used in this study was a purine-requiring strain described by Pearson ('49). It was kept in stock culture on agar slants of the medium employed by Kavanagh ('47).

Prior to examination for decarboxylase activity the bacteria were grown in a complete liquid medium of the following formula:

Peptone	15.0 gm
NaCl	30.0 gm
K_2HPO_4	1.0 gm
$MgSO_4 \cdot 7H_2O$	0.5 gm
L-Amino acid	1.0 gm
Dextrose	5.0 gm
Distilled water	1000.0 ml

Cells were grown in this medium at room temperature (23–25°C.) for a period of time determined by the experiment to be performed and were harvested and washed by centrifuging. Determinations of decarboxylase activity were generally made immediately, but storage of cells at 5°C. for two to three days had no appreciable effect on the decarboxylase activities.

The Warburg manometric method was used for the detection and measurement of the CO_2 which resulted from the decarboxylation of particular amino acids. This well known method is described in detail by Gale ('40).

Generally 1 ml of the bacterial suspension, 1 ml of buffer and 0.5 ml of distilled water (1 ml in the control) were added to the main compartment of the vessel; and 0.5 ml of a 0.033 M solution of the amino acid was pipetted into the side bulb. The 0.05 M buffer solutions were made up from stock solutions of acid potassium phthalate and acid potassium phosphate adjusted with HCl or NaOH to give pH values covering the range 3.5 to 8 inclusive. Anaerobiosis was established by slowly flushing the vessels with nitrogen during the equilibration period of 12 minutes. After equilibration at 28°C. the amino acid solution was tipped into the main compartment, and readings were taken at regular intervals.

In most of the determinations the pH value of the vessel contents was sufficiently acid to cause a direct liberation of CO_2 into gas phase whereas, in others, it was necessary to tip in 0.2 ml of saturated citric acid solution from another side-bulb at the end of the incubation period to liberate bound CO_2.

The separation of the products formed by decarboxylation was effected by paper chromatography. The general method employed was the "ascending method" described by Williams and Kirby ('48).

In preparation for chromatography the contents of the Warburg vessels were centrifuged in order to remove the organisms. Single dimensional chromatograms were run on cylinders of Whatman no. 1 filter paper at room temperature (23–25°C.) using a phenol-water solvent. For comparison, amino acids made up as 0.01 M solutions in 80% ethanol were used. The chromatograms were run for 24 hours, at the end of which time they were dried and sprayed with a mixture of 0.2% ninhydrin in n-butanol. They were developed for one hour at 65°C. in an electric oven; and the spots which appeared were immediately encircled with pencil, since fading occurred within a few days. Variations of this procedure will be discussed in connection with the particular experiment involved.

RESULTS

The following amino acids were examined for possible decarboxylation by washed bacterial suspensions at pH values of 5, 6, and 7: glycine, DL-alanine, DL-valine, DL-tryptophane, L-aspartic acid, L-tyrosine, L-arginine, L-histidine, L-glutamic acid, DL-lysine, L-cystine, and DL-ornithine. CO_2 in measurable amounts was evolved only from L-lysine and L-glutamic acid. Since the tests were of short duration and only Q_{CO_2} values greater than 1 were considered, it is possible that extremely slow decarboxylations were not detected.

The optimum pH for the decarboxylation of glutamic acid was found to be 6 as indicated in figure 1. A slight variation in this pH optimum from experiment to experiment was proba-

bly due to small differences in the condition of the culture. Tests to determine specificity, optimum concentration of substrate, and temperature effects were made at this pH. Generally one to two milligrams dry weight of organisms were needed per Warburg vessel to obtain completely decarboxylation within an hour's time.

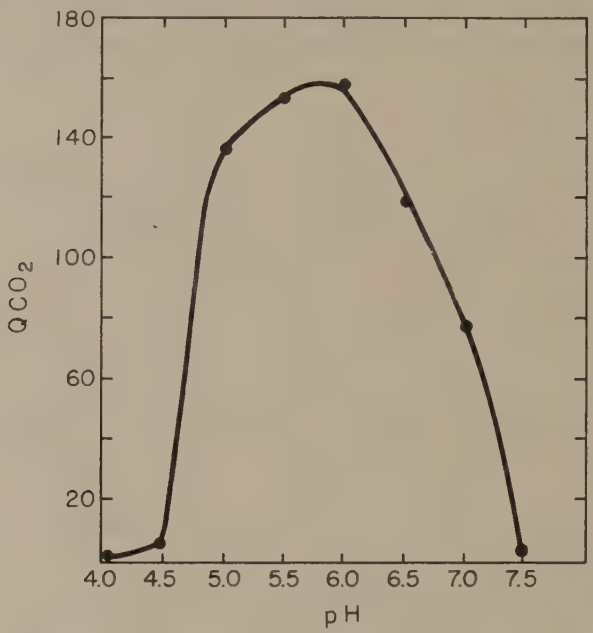

Fig. 1 Variation of glutamic decarboxylase activity (Q_{CO_2}) with pH.

Under the proper conditions of cell mass, pH, etc., the reaction proceeded quantitatively and 92–96% of the glutamic acid was decarboxylated within a 20 to 30 minute period. Data from such an experiment are presented in figure 2. However, the activities of different cell harvests were found to vary considerably and at times the reaction took place at a much slower rate. In some determinations CO_2 evolution was not complete until after one to two hours; in such instances the quantitative yields were usually poor (70–80%).

The Michaelis constant of glutamic acid decarboxylase was found to be approximately 0.0011 M. This value was obtained

by setting up a series of vessels containing washed cells in the main vessel and 0.5 ml of graded concentrations of glutamic acid in the side bulb. After equilibration the substrate was tipped in, and the total CO_2 evolved in 10 minutes was measured. The Michaelis constant was then determined graphically as shown in figure 3.

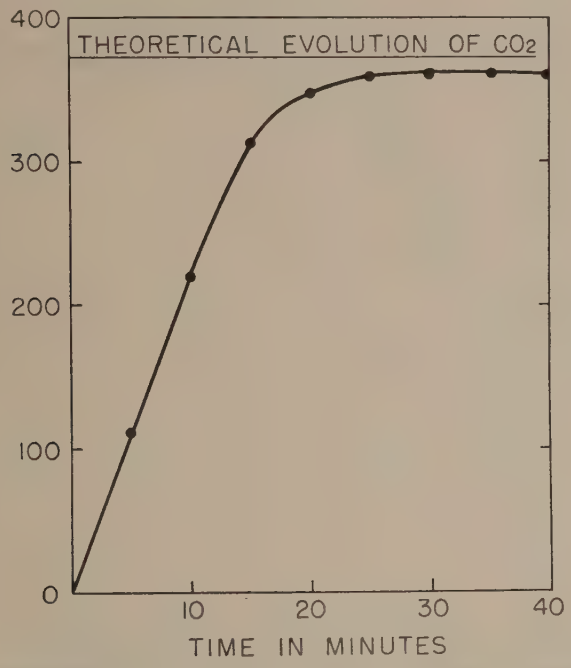

Fig. 2 Quantitative decarboxylation of 0.5 ml of 0.033 M glutamic acid by washed suspensions of *P. fischeri* at pH 6.

Washed suspensions of bacteria did not attack D-glutamic acid or L-glutamine. L-glutamic acid and β-hydroxyglutamic acid were decarboxylated. The latter was decarboxylated so slowly that after three hours only 25% of the theoretical CO_2 production had taken place.

Within the temperature range 20–35°C. the decarboxylation of glutamic acid increased steadily with the increase of temperature. Calculation of the Q_{10} between 20° and 30°C. gave an expected value between 2 and 3.

The production of glutamic decarboxylase by cultures of *P. fischeri* was found to vary a great deal depending upon the age of the culture at the time of harvesting as shown in figure 4. Cells harvested during the lag phase of growth produced little CO_2 from a glutamic acid substrate; but the rate of evolution of CO_2 increased during the logarithmic phase,

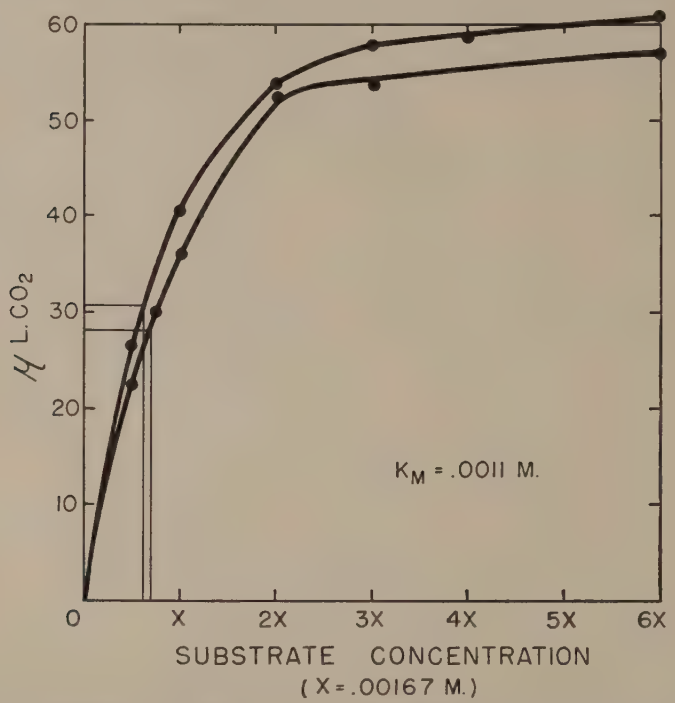

Fig. 3 Variation of initial rate of decarboxylation (μl CO_2) with concentration of glutamic acid: determination of the Michaelis constant.

reached a maximum in the stationary phase (50–60 hours), and then began to decline.

The decarboxylase activities of washed cells taken from cultures incubated at various temperatures showed that optimum production of glutamic decarboxylase takes place at lower temperatures, around 15–20°C. However, in subsequent experiments the cultures were grown at room temperature (23–25°C.) since this is more nearly optimum for the growth of

this strain; and at this temperature the decarboxylase production is still relatively high.

Determinations made with cells grown in a medium containing NH_4Cl as the main nitrogen source showed that decarboxylation took place but did so at a slower rate than by cells grown in the presence of glutamic acid. The fact that glu-

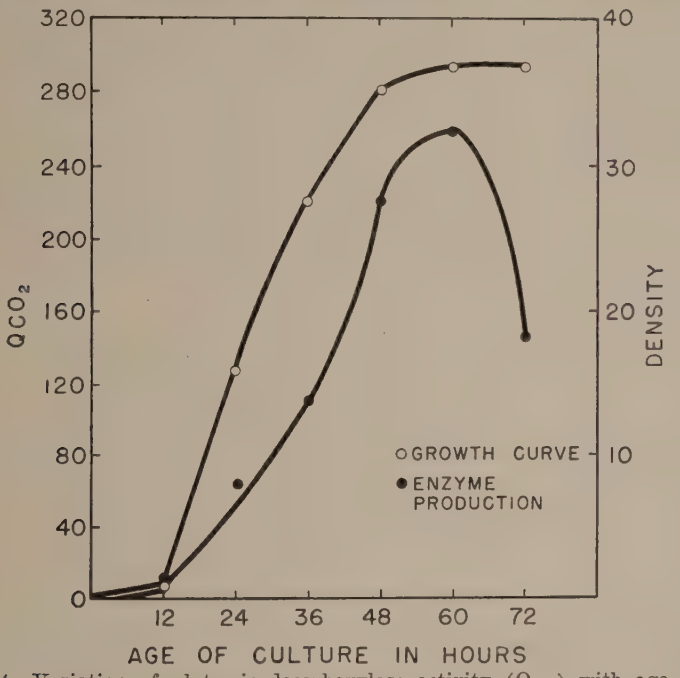

Fig. 4 Variation of glutamic decarboxylase activity (Q_{CO_2}) with age of culture.

tamic acid is not absolutely necessary for the production of glutamic decarboxylase indicates that it is a constitutive enzyme. Cells grown on a peptone agar medium decarboxylated glutamic acid more slowly than those from a peptone broth, thus indicating that aerobic conditions are less satisfactory for the production of this enzyme than semi-aerobic conditions.

One-dimensional ascending chromatographic separations were performed on the contents of the Warburg vessels af-

ter decarboxylation had occurred. In addition to the glutamic acid spot there appeared a new spot at an approximate Rf value of 0.80. By comparison with the Rf value given by Dent ('48) this new spot was tentatively identified as γ-aminobutyric acid. Accordingly, known mixtures of γ-aminobutyric acid, α-aminobutyric acid, and glutamic acid were chromatographed simultaneously with samples of the reaction mixture. The Rf values of the decarboxylation product and γ-aminobutyric acid were identical. This was considered sufficient evidence to identify the material as γ-aminobutyric acid.

Attempts to obtain glutamic acid decarboxylase in a cell-free form were unsuccessful. However, the existing techniques and equipment did not permit the culture and the harvest of the large quantities of cells necessary for an adequate study of this phase of the problem.

Properties of lysine decarboxylase

Washed suspensions of *P. fischeri* were found to decarboxylate L-lysine but not D-lysine. Compared to glutamic acid decarboxylase, the production (or activity) of this enzyme was rather low. In order to obtain enough cells for one determination (5–7 mg dry weight of cells per Warburg vessel) it was necessary to use the bacteria harvested from medium containing 1 gm of L-lysine per liter. Bacteria grown on a medium containing 0.5 gm of L-lysine per liter exhibited very low activity, and cells grown in the absence of added lysine showed no measurable activity. In the initial experiments in which activity was tested in 0.05 M buffer, extremely erratic responses were obtained. This difficulty was partially eliminated through the use of a 0.025 M buffer solution.

The most striking feature of lysine decarboxylase activity is that under the experimental conditions it has no sharply defined pH-optimum as does glutamic decarboxylase. Usually the greatest production of CO_2 occurred at pH range of 5–8. There was also considerable variation in the pH-optimum from experiment to experiment. This variation cannot be accounted for at present.

Chromatograms of the products of lysine decarboxylation were performed in a phenol-H_2O solvent in the presence of ammonia vapors. Such chromatograms revealed two rapidly-travelling spots, one at an Rf value of about 0.90 and the other at about 0.70. Chromatograms of mixtures of known compounds showed that the spots in question were probably cadaverine (Rf 0.90) and lysine (Rf 0.70). Decarboxylation products which were formed at pH values of 5, 6, 7 and 8 were chromatographed in an NH_3 atmosphere and gave similar separations.

Chromatograms of the same mixtures in the absence of NH_3 gave entirely different pictures. Chromatograms of the products of the lysine decarboxylation reaction occurring at pH 5 yielded three definite spots with Rf values of 0.10, 0.25, and 0.50 respectively. In contrast, chromatograms of the products of lysine decarboxylated at pH values of 6, 7 and 8 and gave only two spots (Rf 0.10 and Rf 0.25). Chromatograms of lysine-cadaverine mixtures under these conditions streaked out so that identification of the components was impossible. The identity of the ninhydrin reactive spots at Rf 0.50 was not established. The spots occurring at Rf values 0.10 and 0.25 are probably lysine and cadaverine; the order of occurrence is not known.

Further work on the nature of the decarboxylation of lysine by this organism is in progress.

DISCUSSION

For the most part the general properties of the glutamic decarboxylase occurring in *P. fischeri* are similar to those found in other bacteria (Gale, '41, '46). The properties of lysine decarboxylase have not been investigated thoroughly enough to permit classification.

Glutamic acid seems to play an important part in the metabolism of *P. fischeri*. It has been demonstrated by this author (unpublished data) and by Farghaly ('50) that glutamic acid may serve as a sole source of nitrogen for growth. It has also been shown by Pearson (unpublished data) that

glutamic acid is rapidly oxidized by washed cells of this organism, and evidence has been presented for the decarboxylation of this amino acid to from γ-aminobutyric acid and CO_2.

Farghaly ('50) made the interesting observation that glutamic acid is the only single amino acid capable of supporting the growth of *A. fischeri* in CO_2-free air. He suggests that "the compounds which initiate growth in CO_2-free atmosphere do not actually substitute for CO_2 but merely affect the metabolism of the organism in such a way that the requisite amount of CO_2 is produced." The fact that small amounts of CO_2 are required for the growth of certain microorganisms has been known for many years (Valley and Rettger, '27). Lwoff and Monod ('47, '49) and Ajl and Werkman ('48, '49) have shown that *A. aerogenes* and *E. coli* grow in the absence of CO_2 if members of the Krebs or Szent-Györgyi cycles are present in the medium. Ajl and Werkman ('48) presented results which indicated that the compounds added did not function merely by supplying CO_2 to the organism. Later ('49) these authors considered the mechanism of the substitution and concluded that "all compounds replacing CO_2 function by yielding keto-acids which in turn by amination, transamination, or similar reactions serve as substitutions for carbon dioxide." Farghaly's results ('50) indicated that this was not true for *P. fischeri* because certain members of the Krebs and Szent-Györgyi cycles (pyruvate, malate, fumarate, citrate and α-ketoglutarate) did not replace the CO_2 requirement of this organism, but glutamic acid was able to do so. There is evidence that this replacement was not due to the conversion of glutamic acid to α-ketoglutarate by oxidative metabolism as proposed by Ajl and Werkman ('49), since α-ketoglutarate did not replace CO_2. On the basis of our experiments it is tempting to believe that the decarboxylation of glutamic acid by *P. fischeri* actually supplied sufficient CO_2 to support growth.

In an evaluation of the methods used by Farghaly ('50) it appears probable that the inoculum he used contained the

enzyme, since his inoculum consisted of 0.5 ml of a rather thick suspension of bacteria which had been grown on a peptone agar medium. Low levels of glutamic decarboxylase are produced by this organism even when grown in the absence of glutamic acid.

This raises the question of the biological role of the amino-acid decarboxylases. Since bacteria produce decarboxylases in response to growth in an acid environment, Hanke and Koessler ('24) suggested that the amines resulting from decarboxylation of amino acids function as protective mechanisms against the accumulation of excess H-ions in the medium. The bacterial amino acid deaminases are produced and function best under alkaline conditions. These enzymes catalyze the degradation of amino acids to acids and ammonia and consequently cause the medium to become more acid. These facts, together with the observation that the deaminases and decarboxylases are not produced until after the logarithmic phase of growth, caused Gale ('46) to believe that these enzymes have no important function during active cell division or in the anabolic phase of the metabolism but are merely protective devices which serve to enable the cell to live by partially stabilizing the pH of the external environment.

In view of the fact that the lysine decarboxylase of *P. fischeri* apparently functions as well under alkaline as under acid conditions, it is difficult to accept the "protective mechanism" function as proposed above. It would be unwise, however, to draw any definite conclusions until the nature of this enzyme and its products are more carefully elucidated.

Since it is probable that all bacteria require CO_2 in small amounts for growth, Gale ('46) believes that the amino acid decarboxylases may have a further function in that they may act as CO_2 sources in the medium. The present investigation and that of Farghaly ('50) support this hypothesis. Further substantiation awaits a comprehensive study of the effect of amino acids upon the CO_2 requirement of those organisms known to possess decarboxylases and those which lack such enzymes.

SUMMARY

A purine-requiring mutant of *P. fischeri* possessed enzymes capable of decarboxylating glutamic acid and lysine. Studies with washed cell suspensions showed that glutamic acid decarboxylase acted on its substance to form γ-aminobutyric acid and CO_2. This enzyme, which was found to be constitutive in nature, was rather specific for L-glutamic acid; it did decarboxylate β-hydroxyglutamic acid slowly but failed to catalyze the decarboxylation of either L-glutamine or D-glutamic acid. The optimum activity of this enzyme occurred at pH 6, and under these conditions the K_m value was 0.0011 M. Incubation in a liquid medium at temperature 15° to 20°C. was most favorable for its production. Maximum amounts of this enzyme existed in the cells of a culture at about the time of the cessation of cell division.

Less extensive studies were made with lysine decarboxylase which was found to be adaptive and to catalyze the decarboxylation of lysine to cadaverine, CO_2 and at least one unidentified product. No definite pH optimum was established for this enzyme.

ACKNOWLEDGMENT

The interest and guidance of Dr. C. S. Shoup and Dr. Ilda McVeigh are gratefully acknowledged.

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CYTOCHEMICAL STUDIES OF THE ACTION OF TRYPSIN

II. ANALYSIS OF THE SWELLING OF SALIVARY-GLAND CELLS [1]

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FOUR FIGURES

The first article of this series presented an analysis of the process of structural deformation effected in salivary-gland chromosomes of *Drosophila melanogaster* by the action of buffered solutions of trypsin (Kaufmann, '52). Evidence obtained in that study indicated that such deformation is attributable to swelling of nucleic acids or degraded nucleoproteins in the presence of electrolytes as a consequence of the hydrolysis, although not the dissolution, of chromosomal proteins. A considerable number of the experiments involved treatment of intact salivary glands, and it was found in such preparations that swelling was not restricted to the chromosomes but occurred throughout the cell. An opportunity was thus presented to study the swelling reaction quantitatively by measuring changes in volume of cells and nuclei during the course of treatment. The results of such studies, during which the materials under treatment were being observed continuously with the phase microscope, are presented in this article.

A critical appraisal of the mode of operation of solutions of trypsin in effecting cellular deformation requires an evaluation of the action of the enzyme molecules and the associated

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electrolytes when used independently and in succession, as well as in combination (Kaufmann, Gay and McDonald, '49; Kaufmann et al., '49, '50, '51). One approach to this problem was made in the present study by treating cells with a salt-free, aqueous solution of trypsin at pH 6 (designated hereafter as aqueous trypsin), and then with water and electrolytes at this pH. The sequence of treatments involved hydrolysis with the enzyme, thorough rinsing in water, replacement of the water by a solution of electrolytes, and finally replacement of the electrolytes by water (designated hereafter as the enzyme-water-buffer-water series).

METHODS AND MATERIALS

In order to record accurately the changes in cell shape and volume induced by successive application of a series of reagents, it was necessary to adopt a method that would permit individual cells to expand or contract without becoming displaced from the field of microscopic observation. This requirement was satisfied in the following manner.

Salivary glands were dissected from third-instar larvae of *D. melanogaster* into physiological salt solution (7.5 gm NaCl, 0.35 gm KCl, 0.21 gm $CaCl_2$ per liter distilled water), and transferred while immersed in this medium to a treatment chamber. This was prepared by elevating a cover slip, with strips of Parafilm or paper impregnated with petroleum jelly, above a slide into which a pair of grooves or troughs had been cut. The grooves facilitated the exchange of fluids across the treatment chamber. A rapid and efficient method of exchange involved the removal of liquid at one edge of the cover with a thin strip of absorbent paper and introduction of the replacing fluid at the opposite edge with a medicine dropper.

The depth of the treatment chamber was determined by the thickness of the strips of Parafilm or paper, which ranged from 40 to 100 μ in the experiments reported here. In the shallower chambers (40–50 μ) the broader terminal portions of the salivary glands were held firmly between slide and

cover, so that the preparations were not displaced by the exchange of liquids. Even in the 100 μ chamber, the preparation was held firmly if it included the bulky head and thoracic segments of the larva, the paired salivary glands remaining attached to the pharynx. It was possible to determine in such preparations — using a Spencer phase microscope equipped with an ocular micrometer — the changes that occurred in dimensions of cells and nuclei throughout the course of an experiment. The relatively transparent cells in the narrow anterior portion of the gland were especially useful in these studies (fig. 1). When subjected to experimental conditions that induce swelling, these cells can expand freely until they fill the depth of the treatment chamber. If swelling continues, however, and the cover slip is held firmly by the Parafilm or petroleum-jelly seal along its upper and lower edges, further expansion will be restricted to the lateral diameters, which can be measured with the ocular micrometer, and a disproportionately high estimate of the increase in volume will be obtained. In efforts to detect and avoid this type of error, each of the experiments reported was performed independently by at least two of the authors, who measured 10 or more cells and nuclei in each of a number of different glands, using treatment chambers of different depths. Although the extensive data obtained in this way served to establish the reliability of the method of assay, the changes in volume reported are regarded as approximate rather than absolute, and for that reason have not been endowed with the finality of statistical treatment. Estimates of volume increases given in succeeding pages are based on measurements of entire cells, except in those specified cases in which there was a marked difference in the relative amount of swelling of the nucleus and of the cell as a whole.

Unless otherwise specified all treatments were carried out at 37°C. The slide was kept during the period of treatment on the stage of the microscope, which was enclosed in a plastic box heated by circulating air, whose temperature was thermostatically controlled.

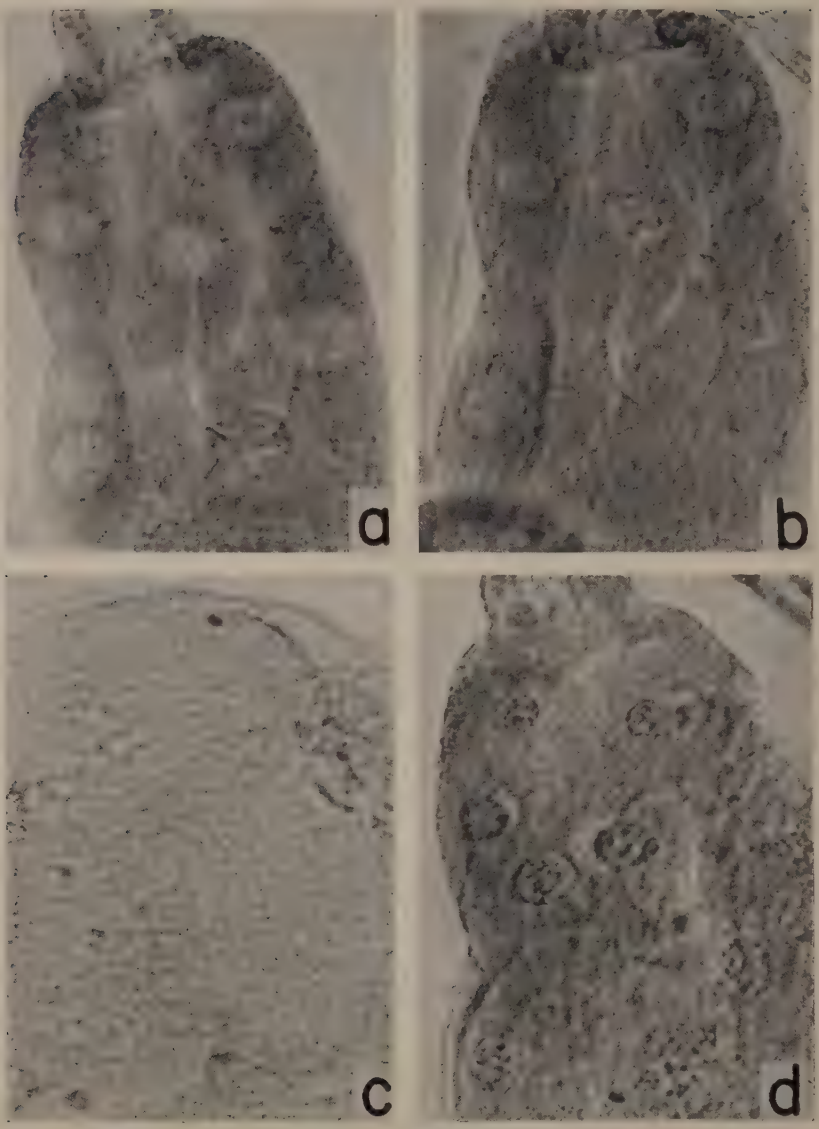

Fig. 1 Swelling of salivary-gland cells induced by application of the trypsin-water-buffer-water series of reagents. (*a*) Cells fixed in aceto-carmine. The same cells (b) after thorough rinsing in water, treatment with trypsin, rinsing in water, and treatment with potassium phosphate buffer (all at pH 6); (*c*) after subsequent application of water, which leads to swelling and loosening of structural frame; and (*d*) after final addition of 45% acetic acid.

The enzymes used included trypsin, chymotrypsin, pepsin, ribonuclease, and desoxyribonuclease. All were extracted, crystallized, and purified in this laboratory. Trypsin, prepared according to the method of McDonald and Kunitz ('46), was dialyzed to remove the inorganic salts used in its crystallization, and was lyophilized from the frozen state. Chymotrypsin, crystallized by the method of Kunitz and Northrop ('35), was also dialyzed and lyophilized from the frozen state. In the course of the studies it seemed desirable to determine the action of chymotrypsinogen; and the sample used, prepared by the method of Kunitz ('48), was purchased from the Worthington Biochemical Laboratory. Unless otherwise stated these materials were dissolved in ice-cold water (2°–4°C.) just before they were to be used cytochemically, and were adjusted with 0.01 N sodium hydroxide to pH 6 as determined colorimetrically on a spot plate using bromcresol purple as an indicator. Chemical assay showed that the samples of trypsin and chymotrypsin used were free of nucleases. The sample of chymotrypsinogen contained about 1% of chymotrypsin as determined by its capacity to digest hemoglobin. Pepsin was prepared according to Northrop ('46) by recrystallizing twice from alcohol. For cytochemical use this enzyme was dissolved in 0.02 N hydrochloric acid, at about pH 1.4, or dissolved in water and adjusted to a pH of either 4 or 5.6. Ribonuclease was dissolved in distilled water and adjusted to pH 6; desoxyribonuclease dissolved in 0.003 M magnesium sulfate and adjusted to pH 6. The methods of preparation and the enzymatic properties of these nucleases are described elsewhere (Kaufmann, Gay and McDonald, '51).

The solvents for the different enzymes were used alone in treating control preparations during the period of time that the experimental preparations were being subjected to enzymatic hydrolysis.

EXPERIMENTAL RESULTS

Effect of different reagents on swelling. Cells of salivary glands of Drosophila obtained by dissecting the larvae in

physiological salt solution were clearly discernible with the phase microscope. The dimensions of cells and nuclei could be measured accurately, and the chromosomes identified by their characteristic patterns of banding. The cells expanded to about 1.6 times their original volume when the salt solution was replaced by distilled water or a salt-free aqueous solution of trypsin, and shrank again when restored to the salt solution. Only slight swelling occurred when a buffered solution of trypsin replaced physiological saline and was in turn replaced by a solution of electrolytes. Unfixed cells were not digested in short periods of time by trypsin in buffered solutions at pH 6 or 7.5, presumably because of failure of the enzyme molecules to enter the living cell and attack native proteins.

Fixed cells were rapidly digested by trypsin. Among the fixatives tested that did not inhibit enzymatic activity were acetic acid, acetic acid-alcohol (1:3), aceto-carmine (aceto-orcein completely suppressed action of the enzyme), and Bouin's fluid. The greatest swelling (upon application of the trypsin-water-buffer-water series) was observed in cells that had been fixed in acetic acid. Dehydration of such cells through graded alcohols, and subsequent treatment with ether-alcohol at 37°C. (for removal of neutral fats), did not inhibit swelling. Fixation in 45% acetic acid was accordingly adopted as a standard procedure in the experiments reported.

When acetic acid replaced physiological salt solution, swelling occurred until the cells had approximately doubled their original volume. The extent of this change is represented graphically in figure 2, which also shows the relative increases or decreases in volume obtained in one experiment with a series of reagents used at pH 6 for the periods of time indicated. Successive treatments with distilled water, a 0.05 M solution of potassium phosphate buffer (symbolized by KK), distilled water, an aqueous solution of trypsin (or inactivated trypsin), water, and phosphate buffer effected only slight changes in volume, without effacing structural details. When water was added to the cells after they had been treated with

an aqueous solution of trypsin and then with electrolytes, however, a marked and rapid swelling occurred (as is shown by the solid line in fig. 2), with consequent deformation of the chromosomes and distortion of their pattern of banding.

In contrast with this result, inactivated trypsin did not induce an appreciable amount of swelling when followed by

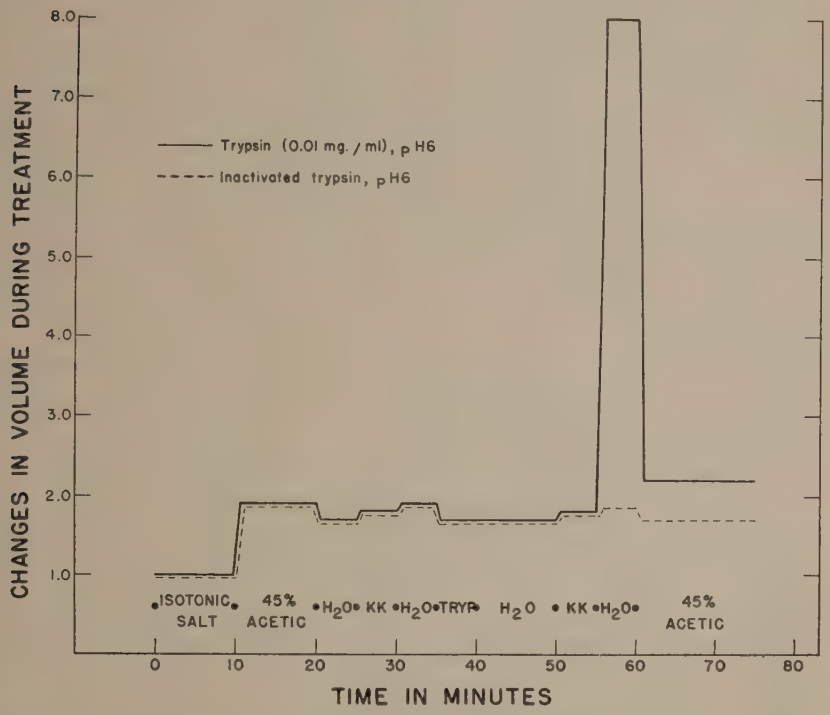

Fig. 2 Changes in volume of salivary-gland cells when treated successively with a series of reagents for the periods of time indicated.

electrolytes and water (as is shown by the broken line of fig. 2), nor did egg albumin, soybean trypsin-inhibitor, or chymotrypsinogen, which were also used to determine the effect of enzymatically inactive proteins of about the same molecular weight as trypsin. The increase in volume obtained in the experiments using chymotrypsinogen was slightly in excess of that obtained with the other two reagents (ca. 5 X

as compared with 2 X), and this is probably attributable to the presence of about 1% of chymotrypsin in the sample of chymotrypsinogen used. Pure crystalline chymotrypsin, on the other hand, induced a 25- to 30-fold increase in volume.

The phenomenon of swelling therefore appears to be dependent on the action of trypsin (or other proteolytic enzymes) on cellular materials. That it is also conditioned by the action of electrolytes is suggested by the fact that cells do not swell appreciably when placed in water after treatment with a salt-free aqueous solution of trypsin, or after successive treatments with electrolytes, water and aqueous trypsin (fig. 2). When non-electrolytes such as glycerine, glucose, and sucrose (in 0.01 M, 0.1 M, and 1.0 M concentrations) were applied to cells that had been digested in an aqueous solution of trypsin, no appreciable swelling was detected on subsequent addition of water, and frequently a decrease in volume was observed.

Effect of concentration of electrolytes and length of treatment. A more precise definition of the action of trypsin and electrolytes in promoting swelling and cellular deformation was obtained by studying the effects of different concentrations of these reagents when used over various periods of time. The studies indicate that the amount of swelling is dependent on the concentration and time of action of both the enzyme and the solution of electrolytes. That the extent of swelling is a function of the time of treatment with the electrolytes is shown by the following results. Salivary-gland cells that had been digested for 10 minutes in 0.01% aqueous trypsin were rinsed in water, and then in 0.05 M potassium phosphate buffer. Upon the subsequent addition of water, it was found that three minutes in the buffer resulted in a 7-fold increase of the original volume of the cells, 5 minutes in a 13-fold increase, 10 minutes a 30-fold increase, 20 minutes a 24-fold increase, and 40 minutes only a 14-fold increase. In a comparable experiment in which digestion in a 0.01% aqueous solution of trypsin continued for 8 minutes, a similar relation of amount of swelling to time in buffer was detected,

although the values were somewhat lower than those indicated above (namely, 6X, 13X, 22X, 10X and 6X, respectively, for periods of 2, 5, 10, 20, and 40 minutes in 0.05 M buffer). The basis for the decrease in effectiveness of the reagent after a maximum is reached will be considered later.

The amounts of swelling obtained with different concentrations of potassium phosphate buffer are shown in figure 3.

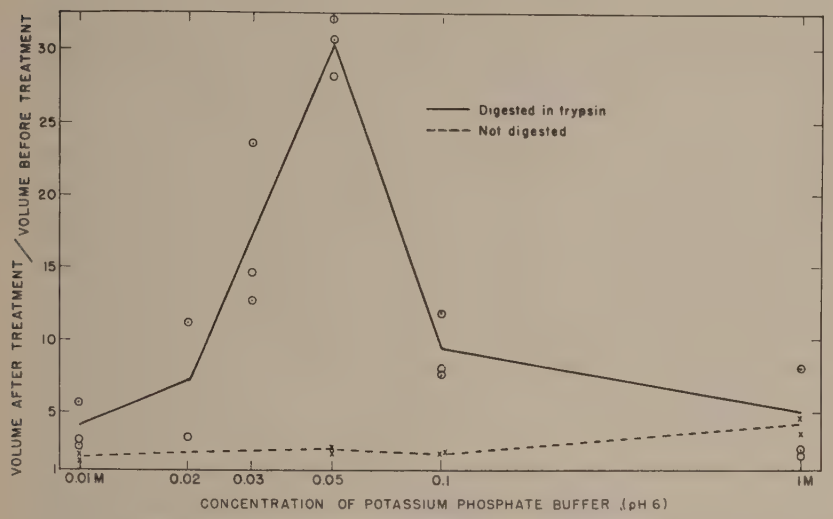

Fig. 3 Effect of concentration of potassium phosphate buffer on swelling of salivary-gland cells. Treatment involved application of the trypsin-water-buffer-water series at pH 6. The solid line represents average values for three experiments, represented by circles; the dotted line for two experiments, represented by crosses.

In the three experiments represented, the cells were treated for 10 minutes with a 0.01% aqueous solution of trypsin, rinsed thoroughly in water to insure removal of uncombined trypsin, treated for 10 minutes with potassium phosphate buffer, and then with water. When relative increase in cell volume is plotted against molar concentration of the buffer, it is apparent that maximum swelling occurred at the 0.05 M level. On the other hand, swelling of cells that had been treated with solutions of inactivated trypsin, as shown by the broken line of figure 3, reached a maximum, which was much

lower than that of the trypsin-hydrolyzed cells, at the highest concentration of the buffer. An effect of concentration of electrolytes was also detected in experiments using sodium chloride, maximum swelling being effected by a 0.1 M solution, although the actual increase in volume was much less than that obtained with 0.05 M potassium phosphate buffer.

The action of various cations and anions. It seemed desirable, therefore, to determine the effect on the swelling reaction in trypsin-hydrolyzed cells of a number of different cations and anions. Efforts to prepare the necessary reagents were restricted by the general plan to use all solutions at pH 6. In conformity with this requirement, one series of cations was tested by using sodium, potassium, ammonium, lithium, calcium, magnesium, strontium, and barium chlorides. Each of the salts was dissolved to give an ionic strength of 0.064, which is essentially that of 0.05 M potassium phosphate buffer at pH 6, and was adjusted to pH 6 with the corresponding base or acid. Treatment in each of the solutions continued for 10 minutes after the cells had been hydrolyzed for 10 minutes in a 0.01% aqueous solution of trypsin. Final measurements were made after water had replaced the solution of electrolytes. The sodium and potassium salts were the most effective members of this series in inducing swelling, although the maximum increase in volume was considerably less than that produced by 0.05 M potassium phosphate buffer. Some of the salts, especially those with divalent ions, caused shrinkage rather than swelling of cells and nuclei.

The anionic series included sodium nitrate, chloride, chlorate, sulfate, acetate, citrate and phosphate. Each salt was dissolved in water to give an ionic strength of 0.064, and adjusted to pH 6. When these reagents were used on cells that had been treated with a 0.01% aqueous solution of trypsin, practically no swelling was obtained with the first three, only slight swelling with the acetate, and a moderate amount with the citrate, but approximately the same amount with the sodium phosphate buffer as with 0.05 M potassium phosphate buffer. These results suggest that the phosphate anion may

be particularly important in promoting the swelling of materials within a cell that has been exposed to the action of a proteolytic enzyme.

Effect of concentration of trypsin and duration of treatment. In the light of these observations, a 0.05 M solution of potassium phosphate buffer was used for 10 minutes in experiments to determine the amount of swelling produced by treatment with different concentrations of trypsin at pH 6 for various periods of time. The results obtained in one series of experiments are shown in figure 4. Preliminary data have been reported (Kaufmann et al., '50) in a similar diagram. Both sets of data indicate that the amount of swelling depends on the concentration of trypsin used and the duration of the treatment. At any one concentration, the amount of swelling reaches a maximum and then falls off rapidly. The highest concentration of trypsin used, which was 1 mg per milliliter (a 0.1% solution) gave the greatest amount of swelling; the lowest concentration used, 0.001 mg per milliliter, gave the least swelling (with the 0.01 and 0.001% concentrations giving intermediate values). The action of trypsin was more rapid the higher the concentration; for example, maximum swelling was induced when a 0.1% solution acted for one or two minutes, but not until a 0.001 or 0.0001% solution had acted for 20 to 40 minutes. That the type of curve represented in figure 4 is not dependent on conditions imposed by successive treatments with aqueous trypsin and electrolytes is indicated by the fact that curves showing similar time-concentration relations were obtained with cells that had been digested in solutions of trypsin in phosphate buffer at pH 6 before being rinsed in water.

On the basis of these findings the experimental procedure was standardized in the following manner. Glands were fixed for 10 minutes in 45% acetic acid, and rinsed for 5 minutes in distilled water. Initial measurements were then made of selected cells and nuclei. The preparations were then hydrolyzed for 10 minutes in a 0.01% aqueous solution of trypsin at pH 6, rinsed for 5 minutes in distilled water,

treated for 10 minutes with 0.05 M potassium phosphate buffer at pH 6, and again rinsed in distilled water for two minutes before the final measurements were taken. Unless otherwise stated, any subsequent reference to the enzyme-water-buffer-water sequence involves the time intervals and concentrations indicated here.

The reversible nature of the swelling process. Trypsin-induced swelling is a reversible process. If a solution of

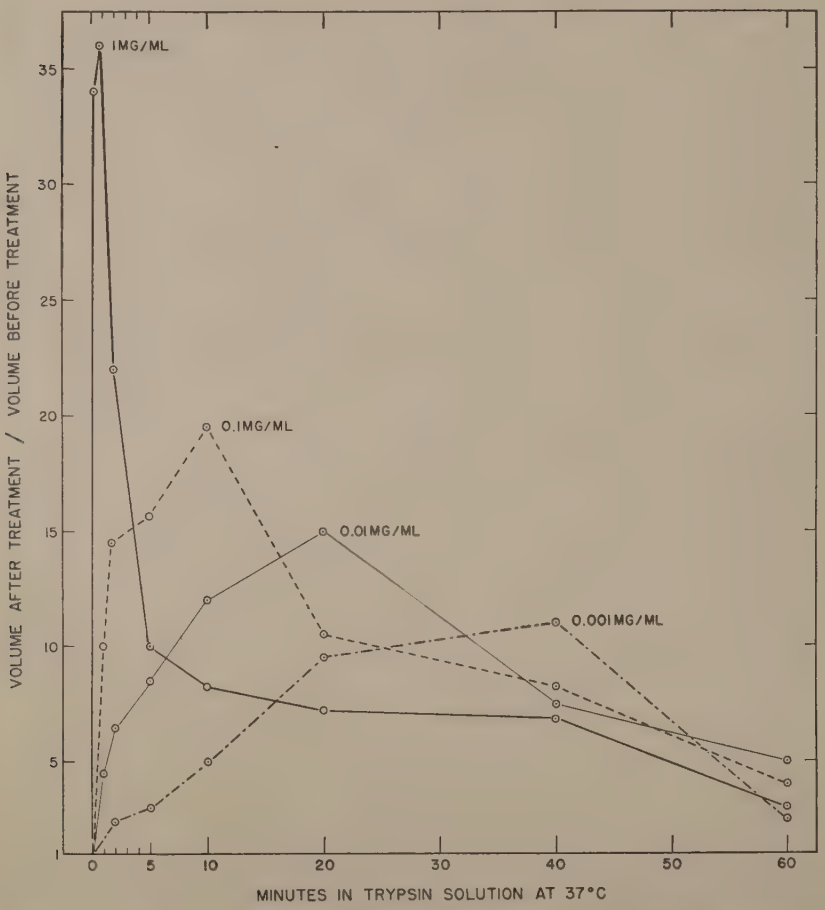

Fig. 4 Effect of concentration and time of action of aqueous solutions of trypsin on swelling of salivary-gland cells. Treatment included the trypsin-water-buffer-water series, all reagents being used at pH 6.

electrolytes, such as 0.05 M potassium phosphate buffer or 0.1 M sodium chloride, is drawn into the treatment chamber while the cells are swollen, they shrink rapidly. Swelling upon the addition of water, and shrinking when water is replaced with buffer, can be induced repeatedly in the same cell, each time with some additional deformation of structure, as evidenced by changes in the appearance of the bands of the chromosomes. Shrinkage can also be effected by treating the swollen cells with 45% acetic acid. Subsequent treatment with a water-buffer-water series will again cause swelling (fig. 1). In one experiment, cells that had been fixed in aceto-carmine, which retards but does not inhibit tryptic hydrolysis, were subjected 9 times to the swelling-shrinking cycle without disintegrating completely.

Evidence that aqueous solutions of trypsin are enzymatically active. Since the experiments described above showed that the amount of swelling depends on the concentration of both the enzyme and the solution of electrolytes, and on the duration of the treatment with each of these reagents, it was necessary to determine whether trypsin in a salt-free aqueous solution can hydrolyze cellular substrates, or whether hydrolysis is delayed until the electrolytes have been added.

Experiments of a qualitative nature made in this laboratory by Dr. Margaret McDonald have demonstrated that suspensions of salt-free, denatured hemoglobin, egg albumin, and fibrin go into solution when exposed to the action of trypsin at pH 6. Evidence has also been obtained that aqueous solutions of trypsin can degrade the materials within fixed salivary-gland cells. These studies involved comparisons of the extent of swelling when aqueous trypsin (and the other reagents) were used at different temperatures, and when trypsin inhibitors were used before or after aqueous trypsin or combined with it. A general appraisal of all these experiments indicated that trypsin in aqueous solution is capable of degrading substrate materials in fixed cells, although some evidence was obtained that adsorbed enzyme may continue to act after the buffer is added. More extensive data in support of the con-

clusion that aqueous solutions of trypsin are enzymatically active have been obtained in studies on sections of root tips of onion and lily, and a general discussion of the problem will be reserved until those data are published (Gay, in preparation).

Inhibition of swelling by degradation of nucleic acids. The conditions influencing swelling having thus been established, it remained to be determined what materials within the fixed cell were responsible for the marked changes in volume. An important clue was obtained in the analysis of the action of reagents at different temperatures when it was observed that water at 60°C. markedly reduced the amount of swelling as compared with water at 37°C. Since previous studies had shown that water at 60° will degrade nucleic acids in fixed cells (Kaufmann, Gay and McDonald, '49; Kaufmann, '50; cf. Oes, '10; Brachet, '40; Panijel, '47), experiments were undertaken to determine the capacity of cells to swell after removal of the nucleic acids.

The nucleic acids were either degraded by treatment with ribonuclease or desoxyribonuclease, or extracted with perchloric acid or trichloracetic acid. The efficacy of these reagents in removing nucleic acids and thereby degrading nucleoproteins was confirmed by studies of staining reactions in control preparations, using cytochemical methods whose validity had previously been established (see, for example, Kaufmann, '50; Kaufmann, Gay and McDonald, '51; Kaufmann, McDonald and Gay, '51).

The standard procedure for removal of ribonucleic acid (RNA) involved treatment for one hour at 37°C. with a 0.01% aqueous solution (pH 6) of ribonuclease. Desoxyribonucleic acid (DNA) was degraded by treatment of the cells for one hour at 37°C. with a 0.025% solution of desoxyribonuclease in 0.003 M magnesium sulfate (pH 6). Both types of nucleic acid were removed from the same cell by consecutive treatments with the two nucleases, or by a single treatment with either perchloric acid or trichloracetic acid.

Removal of either or both of the nucleic acids by any of these reagents reduced or inhibited the swelling of cells when they were subsequently treated with trypsin, water, buffer, and water. Thus, digestion with ribonuclease inhibited swelling, and often caused marked shrinkage of the cytosome, so that the cell membranes were drawn close to the nucleus. Water, when used at the same temperature and pH as the ribonuclease, had no inhibitory effect. Treatment with desoxyribonuclease retarded swelling, as determined by measurements of entire cells (in one experiment to about one-third of the value obtained in controls treated with 0.003 M magnesium sulfate), but had a more marked effect on the nucleus, in which the DNA is concentrated, actually causing its shrinkage (to about 0.6 of the original volume in the same experiment). When cells were treated successively with the two nucleases, no swelling occurred upon subsequent application of trypsin, water, buffer, and water; and in many cases there was marked shrinkage of both nucleus and cytosome.

Perchloric acid was used in a 5% solution for 15 minutes at either 4°, 37°, or 60°C. Treatment at 4° had little effect on subsequent swelling, as compared with water of the same temperature. Treatment at either 37° or 60°, however, completely inhibited swelling. Trichloracetic acid in 0.3 N solution was tested at 4°, 60°, and 90°C. Treatment at 4° had no effect on swelling, as compared with water; but marked inhibition occurred at 60°. Trichloracetic acid at 90° not only inhibited swelling but actually caused shrinkage of the cells. Water at this temperature also inhibited swelling, but previous studies had shown that water at 90° is effective in degrading nucleic acids. Other controls, involving use of the Feulgen method for detection of DNA, showed that tricloracetic acid at 60° reduced Feulgen-stainability as compared with water of the same temperature, and that a slight amount of Feulgen-stainable material remained after treatment of the cells with water at 90° but no trace of DNA after extraction with trichloracetic acid at this temperature.

The foregoing studies suggest that inhibition of swelling is effected by degradation of nucleic acids. The possibility that the nucleases act to prevent swelling by combining with cellular substrates in a manner that inhibits the subsequent action of trypsin seems improbable, because of the results obtained with perchloric acid and trichloracetic acid. More direct evidence has been obtained by using either ribonuclease or desoxyribonuclease after trypsin, in the following sequence: trypsin, water, nuclease, water, buffer, water. Under these conditions swelling was inhibited to about the same extent as when the nuclease was used before trypsin.

Since trypsin-induced swelling of cells was inhibited by the degradation of nucleic acids, the question arose whether similar inhibition would be effected if the intact nucleic acids were precipitated within the cell. Lanthanum salts were used for this purpose, since lanthanum ions combine with nucleic acids (Caspersson, Hammarsten and Hammarsten, '35). When salivary-gland cells were treated with a 0.01 M solution of lanthanum acetate, swelling, upon subsequent application of the trypsin-water-buffer-water series, was less than in the controls but not completely inhibited. The amount of suppression was a function of the concentration of the lanthanum salt, more swelling occurring after treatment with a 0.001 M solution than after a 0.1 M solution. In one experiment, cells treated with 0.0001 M lanthanum acetate swelled to about 5 times their original volume as compared with 31 times in the controls. In other experiments it was determined that suppression of swelling by lanthanum acetate occurred if this reagent was applied at any stage in the trypsin-water-buffer-water sequence before application of the final water rinse.

Cellular swelling induced by treatment of cells with chymotrypsin and pepsin. Some experimental evidence has previously been presented that swelling of salivary-gland cells is not dependent upon hydrolysis in trypsin, but may also be effected by treatment with other proteases, such as chymotrypsin or pepsin.

No extensive studies were made of the conditions influencing the amount of swelling in chymotrypsin-treated cells, but it was established that this enzyme also causes swelling and cell deformation when used under the conditions described above for trypsin. From the limited data available, it appears that the amount of swelling induced by a given concentration is of the same order of magnitude for both the enzymes.

Pepsin, as ordinarily used in 0.02 N hydrochloric acid at pH 1.4, causes shrinkage of salivary-gland cells without other marked structural deformation (Mazia and Jaeger, '39; Frolowa, '44; Kaufmann, Gay and McDonald, '49). If the shrunken, pepsin-hydrolyzed cells are rinsed thoroughly in distilled water, and are then treated with 0.05 M potassium phosphate buffer and water, marked swelling occurs. As with tryptic hydrolysis, the amount of swelling is a function of the time of treatment with the enzyme. The following values were obtained in one experiment in which a 0.1% solution of pepsin in 0.02 N hydrochloric acid was used for different periods of time before treatment with phosphate buffer and water: three minutes — 6 times the original volume; 5 minutes — 9 times; 10 minutes — 15 times; 20 minutes — 14 times; and 40 minutes — 6 times. The control, which was treated with 0.02 N hydrochloric acid, rinsed in water, and treated with buffer and water, showed no increase in volume. In other experiments, in which pepsin was used at pH 4 or pH 5.6, a similar dependence of the amount of swelling on the time of enzymatic hydrolysis was observed.

DISCUSSION

The experiments reported developed from efforts to determine the mode of action of trypsin in effecting cellular deformation. Cytochemical methods employing enzymatic hydrolysis should constitute one of the most useful means of studying the organization of the cell. They offer the opportunity of determining the pattern of association of cellular materials as well as their chemical nature and location. These

potentialities can only be realized, however, if the precision of the purified enzyme as a tool in "dissecting" the cell is paralleled by precision in the use of this tool and in the interpretation of the information afforded thereby. Analysis of the mode of action of trypsin illustrates the necessity of examining the entire course of the reaction if valid conclusions are to be drawn concerning the relation of enzymatic hydrolysis to total cellular response. It has been shown in the studies reported here, for example, that trypsin *per se* does not effect complete cellular disintegration, but that degradation may ensue because of alterations in the gel-like properties of the degraded nucleoproteins. Attention has been focussed in this way on the importance of the nucleic acids in determining the physical properties of the cell. Although the experiments were performed on fixed cells, it is believed that the results may elucidate, in terms of association of nucleic acids and proteins, some of the alterations in form and structure that occur regularly or are induced in the living cell. It is of interest in this connection to note that the greatest sensitivity of the nucleoproteins of the fixed cell, as determined by their capacity to swell, results from the use of reagents at concentrations and pH levels similar to those to which the nucleoproteins were exposed in the living cell (as if hysteresis were being exhibited at the cellular level). The implications of this type of analogy will be elaborated in another publication; their validity rests upon an understanding of the nature of the swelling reaction, and it is to this problem that the remainder of the discussion will be devoted.

The nature of the swelling reaction. The present study has shown that the products of protease hydrolysis of cellular nucleoproteins have the properties of an elastic gel and are capable of undergoing marked changes in volume as water enters or leaves the cell. Evidence has been accumulated in this and related studies that swelling — and the accompanying deformation of structure — cannot be attributed to dissolution of the hydrolyzed proteins. Trypsin, chymotrypsin, and

pepsin attack different peptide linkages (see, for example, Rovery and Desnuelle, '51), but all of these enzymes elicit similar swelling reactions. The dependence of this response on the presence of nucleic acids in the cell was demonstrated by the retardation of swelling after treatment with nucleases. The effectiveness of treatment with ribonuclease in retarding swelling in nucleus and cytoplasm is consistent with available information that both contain RNA, but the reduction of cytoplasmic as well as nuclear swelling by a sample of desoxyribonuclease that had no measurable traces of ribonuclease raises a question concerning the mechanism involved. It might be assumed that the cytoplasm of these cells contains some DNA, either as a normal constituent or as a post-fixation diffusion product (Kaufmann et al., '50). No support for this assumption has been obtained in our cytochemical studies. Moreover, chemical studies of cytoplasmic fractions from vertebrate cells, such as rat liver, have not revealed any DNA. On the other hand, there is some suggestion that desoxyribose may exist in the cytoplasm of eggs of echinoderms (Vendrely and Vendrely, '49; Mirsky and Ris, '51), and amphibia (Hoff-Jørgensen and Zeuthen, '52), and desoxyribonuclease has been reported to occur in the cytoplasm of embryonic cells of *Arbacia punctulata* (Mazia, '49). Another possible explanation of retardation of swelling of salivary-gland cells by desoxyribonuclease is based on the assumption that the gel forms a network throughout the fixed cell, and that its capacity to swell is reduced by removal of DNA from the nucleus.

Evidence has been presented that nucleic acids and proteins are combined in fixed cells as nucleoproteins, from which the protein moiety can be freed by the action of nucleases (Kaufmann, Gay and McDonald, '51). Results of the present study may be interpreted as indicating that dissociation may also be effected by the converse action, namely, the digestion of proteins and the release of nucleic acids. Whether the dissociation is complete or partial cannot be stated with finality from our experimental evidence, although the efficacy of the

phosphate ions in influencing swelling suggests that they may function in establishing the gel-frame by combining with proteins that remain attached to nucleic acids. (Failure of trypsin to completely dissociate nucleic acid and protein has been reported by Cohen, '45, who found amino acids in dialyzed tryptic digests of desoxyribonucleohistone.) Whatever the extent of dissociation may be, the products formed appear to constitute a gel-network similar to that postulated by Butler and James ('51) for solutions of DNA. Structural viscosity and streaming birefringence of thymus nucleate are reduced by combination with protein, but subsequent removal of the protein restores these properties to a considerable degree (Greenstein and Jenrette, '41). Our data also suggest that the properties of nucleic acids within a fixed cell are modified by dissociation from protein.

It may be assumed that in the degradation of nucleoproteins new sites are uncovered for attachment of available ions. As the ions combine — either upon the addition of electrolytes to a cell after treatment with an aqueous solution of a protease, or during its digestion in a buffered solution — some swelling occurs. But much more swelling takes place upon the subsequent addition of water. This is evidently an osmotic phenomenon, since the cell will shrink if the water is replaced by a solution of electrolytes, and will swell again when water is added. The concomitant alterations in osmotic pressure may be visualized as attributable to a Donnan type of equilibrium, the gel surface acting as a membrane across which the water diffuses because of the high concentration of nondiffusible colloidal ions within the gel network.

Swelling occurs rapidly, the maximum increase in volume being attained within a few seconds. If the increase is not too great, cell and nuclear membranes stretch but do not rupture. One of the more interesting aspects of the phenomenon is the relation of amount of swelling to concentration of electrolytes. This resembles in some respects the effect of salt concentration on the viscosity of nucleic acid, reported by Creeth, Gulland and Jordan ('47). It had been shown by

Greenstein and Jenrette ('41) that the viscosity of solutions of the sodium salt of DNA is reduced by addition of neutral salts. Creeth et al. found that this reduction could be effected by low salt concentrations, and that a critical level was reached at about 0.01 M with sodium chloride, beyond which increased concentrations produced little additional effect. The swelling reaction of the present study differs, however, in the gradual decline in effectiveness of the electrolytes after a maximum has been reached.

The decrease in effectiveness of electrolytes, when used in higher concentrations or for longer periods of time than necessary to produce maximum swelling, finds a parallel in the behavior of the enzyme solutions. Such dependence on time and concentration as is illustrated in figure 4 can be interpreted most readily on the assumption that only the partially degraded material will exhibit the swelling phenomenon. Another possibility is that two contrary processes are represented, one of which leads to swelling, the other to subsequent retardation of the swelling process. The initial slopes of the curves shown in figure 4 suggest that it might be possible to determine the direct relation of swelling to concentration of enzyme if more extensive data were available. Retardation of swelling, which occurred sooner or later with all the concentrations of the enzyme tested, may result from the combination of products of protease hydrolysis with nucleic acids to form compounds incapable of swelling upon the subsequent addition of electrolytes and water.

SUMMARY

Fixed cells exposed to the action of buffered solutions of trypsin undergo marked structural deformation, involving swelling of nucleus and cytosome. An analysis has been made of the swelling reaction in salivary-gland cells of *Drosophila melanogaster* by resolving the process of tryptic digestion into component phases. This was done by hydrolyzing with an aqueous solution of trypsin, rinsing in water, adding electrolytes, and then adding water (all at pH 6). The amount

of swelling was found to depend on the concentration and time of treatment with both enzyme and electrolytes. Of the various anions tested, phosphate was most effective in promoting swelling of the trypsin-hydrolyzed cells; and of the cations tested, sodium and potassium were the most effective. Swelling was inhibited if nucleic acids were removed from the cell, either before or after treatment with aqueous trypsin. These studies show that the products resulting from enzymatic hydrolysis of nucleoproteins have the properties of an elastic gel, and are capable of undergoing marked changes in volume as water enters or leaves the cell. Trypsin *per se* does not effect cellular deformation, which results from alterations in the gel-like properties of the degraded nucleoproteins. Chymotrypsin and pepsin also may be employed under conditions that will ensure swelling. The nature of the phenomenon, and its implications, are discussed.

ACKNOWLEDGMENTS

The authors desire to acknowledge with thanks their indebtedness to Dr. Margaret McDonald for her painstaking efforts in preparing and assaying the enzymes used in these studies, and for her counsel during the progress of the work. They also wish to express their thanks to Major R. C. MacDuffee, U. S. Army Medical Corps, for his assistance during the preliminary stages of the study, and to Miss Helen Gay and Dr. Kenneth Paigen for many helpful suggestions.

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DIURNAL RHYTHM IN THE REGULATION OF DISTAL RETINAL PIGMENT IN PALAEMONETES

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SIX FIGURES

INTRODUCTION

Light- and dark-adaptation of the crustacean compound eye includes movements of the retinal pigments. Although illumination is known to be an important factor in determining the position of the retinal pigments, the mechanism which controls pigment migration is not fully understood. The widespread occurrence of persistent diurnal rhythm of one or more of the retinal pigments indicates that factors other than light must be taken into account. In 1930 Welsh described a persistent diurnal rhythm of retinal pigment migration for the prawn, *Macrobrachium*. He suggested that both nervous and hormonal mechanisms might be involved.

Numerous reports of persistent diurnal rhythms of various retinal pigments have appeared. Summaries of reported retinal pigment rhythms (Brown, '44; Kleinholz, '49) show that any one or almost any combination of pigments may be involved in the persistent rhythm. In some cases a rhythm persists in constant light, in other cases only in constant darkness, and in some cases the rhythm persists both in constant light and constant darkness.

[1] This investigation was supported in part by a research grant from the Graduate School of Northwestern University.

Knowles ('50) reported his investigations on *Leander serratus*. He found that there is a diurnal rhythm of the proximal and reflecting pigments which persists in constant darkness. After removal of the sinus glands the rhythm was still present. He further reported that migration of the proximal pigment in response to light was unaffected by sinus gland removal. The effect of sinus gland removal on the distal pigment, which does not show a persistent rhythm, was less clear. In some of the operated animals the distal pigment remained in the dark-adapted condition regardless of illumination; in others some movement toward light-adaptation was observed. In no case was complete light-adaptation of the distal pigment obtained in the absence of the sinus gland.

It is obvious that a relatively complex mechanism is required to account for all of the results that have been reported. The influence of light might be mediated by nervous mechanisms, by nervous and hormonal mechanisms, or directly on the pigment cells acting as independent effectors. A purely nervous mechanism was ruled out by most investigators on the basis that motor innervation of the pigment cells had never been demonstrated. The demonstration of some degree of interrelation between the two eyes of an individual and the frequent occurrence of persistent diurnal rhythms precluded an explanation solely in terms of independent effector activity.

Kleinholz ('36) and Welsh ('39) presented evidence, from injection experiments, that a light-adapting hormone arising in the eyestalks, and principally the sinus glands, was responsible for inducing certain retinal pigments to move to the light-adapted position.

The first evidence for the presence and normal operation of a dark-adapting hormone was presented by Brown, Fingerman, and Hines ('52) and Brown, Hines, and Fingerman ('52). These investigators concluded, on the basis of injection experiments and the influence of various factors on the rates of pigment changes, that two antagonistic hormones operated in control of the distal retinal pigment of *Palaemonetes*.

It had long ago been pointed out by Welsh that the retinal pigments of crustaceans offered favorable material for study of the physiological basis for diurnal rhythms. Welsh ('41) had shown that the diurnal rhythm of pigment migration in *Cambarus* persisted with the same frequency in animals maintained at 7°C. as in animals at 21°C. A similar temperature-independence over a temperature range from 6°C. to 26°C. was reported by Brown and Webb ('48) for the diurnal rhythm of the integumentary chromatophores of *Uca*. These investigators found that at 0°C. the diurnal rhythm was delayed by an interval approximately that of the time of exposure to this low temperature. They concluded that the diurnal rhythm was metabolically determined, at least in part.

Smith ('48) found that the retinal pigment rhythm of certain grapsoid crabs was inhibited by constant illumination but was re-established upon return of the animals to the dark. When the constant illumination was ended at 7 A.M. Smith observed that the re-established rhythm was 6 hours out of phase with the normal. A similar observation was made independently for the diurnal rhythm of integumentary chromatophores in *Uca* by Brown and Webb ('49). On the basis of this and other evidence Brown and Webb concluded that a basic center of rhythmicity continued to be active in a rhythmical manner even in the absence of the normally observed expression of the rhythm. Further characteristics of the rhythm of sensitivity to light exhibited by the basic center of *Uca* were reported by Webb ('50), and it was suggested that the rhythms previously reported for several other animals were capable of interpretation in terms of a similar rhythmical mechanism.

The investigations reported here grew out of the study of the conditions of illumination responsible for variations in the rate of pigment migration in *Palaemonetes vulgaris*. It soon became clear that a diurnally rhythmic process, not directly induced by illumination, was also contributing to the variations observed. Experiments were then designed to permit a description of the character of the diurnal rhythm.

MATERIALS AND METHODS

All of the animals used in these experiments were specimens of *Palaemonetes vulgaris,* collected at Woods Hole, Massachusetts, during the summer of 1951. Both male and female animals were used. Animals were collected about every third day and at the time of one collection animals from the previous collection were discarded. Stock animals were kept in the laboratory in aquaria supplied with running sea water.

The animals to be used in an experiment were separated into groups of 10 animals and each group placed in a circular pan, 7 to 8 inches in diameter, containing sea water about an inch deep. For those experiments in which an illumination of 250 ft. c. was used, the animals were placed in white enamelled containers in a room which was not exposed to outdoor illumination. The desired illumination was obtained by means of an incandescent lamp at an adjusted distance above the containers. When animals were to be exposed to darkness a photographic darkroom was used. When a black background was to be used, the animals were placed in circular aluminum pans painted with black enamel on the inside.

The state of the distal retinal pigment was determined in the following manner. Animals were held, ventral side down, on the stage of a dissecting microscope and viewed with transmitted light. Under these conditions a distal clear area is visible in the eye in light-adapted animals. The proximal edge of the clear area marks the outer limit of the distal retinal pigment. By means of an ocular micrometer the width of the clear area from its proximal limit to the surface of the eye was measured. A second measurement, from the surface of the eye to the dorsally located "ocellus" was also made. Both measurements were made as nearly as possible along the long axis of the eyestalk. The value of the first measurement decreases as the animals become dark-adapted while the second, of course, remains constant for any one animal. The value of both measurements varies with the size of the animal but the ratio of the first to the second varies principally with the degree of light-adaptation. Therefore

this ratio is used as an index of light-adaptation. This distal pigment index for any one group is obtained by averaging the ratios of the 10 individuals.

The value of the distal pigment index so obtained for fully light-adapted animals was about 0.2, while that for fully dark-adapted animals was 0. When dark-adapted animals are exposed to a bright light for one minute and then returned to darkness and examined at arbitrarily selected intervals after stimulation, a rough measure of the amplitude and duration of the response can be obtained by summing the averages of the several determinations. Although it is theoretically possible that the values obtained in this manner could be similar for two tests in one of which low amplitude and long duration occurred while in the other the response was of high amplitude and short duration this difficulty never arose in practice. This method of comparison was used only when animals from constant darkness were exposed to one minute of light and under these conditions it was found, in general, that a high amplitude of response was accompanied by relatively long duration and when the amplitude was smaller the duration was shorter. It will be seen, however, that the differences amongst the experiments were determined predominantly by duration, that is, differences in rates of re-dark-adaptation.

EXPERIMENTS AND RESULTS

1. Influence of duration of light period on rate of dark-adaptation. For these experiments a large group of animals (over 200) was dark-adapted overnight and the experiments were started the following morning at 8 A.M. For each exposure time 5 groups of 10 animals each were illuminated simultaneously at 250 ft. c. The average state of the retinal pigment of one group was determined at the end of the period of illumination and then all 5 groups were placed in the dark. At the end of 30 minutes in the dark one group of 10 animals was brought into the light and the state of the retinal pigment determined immediately. Determinations were similarly

made after 1, 2, 3, and 4 hours in the dark. The durations of the light periods were as follows: 40 minutes, 90 minutes, 2 hours, 4 hours, and 6 hours.

The results of these experiments are presented graphically in figure 1. Examination of the figure shows that in no case is dark-adaptation complete during the period of observation. Furthermore, dark-adaptational rate, as indicated by the slopes of the curves, does not in any case reach the maximum rate observed under some other conditions. A compari-

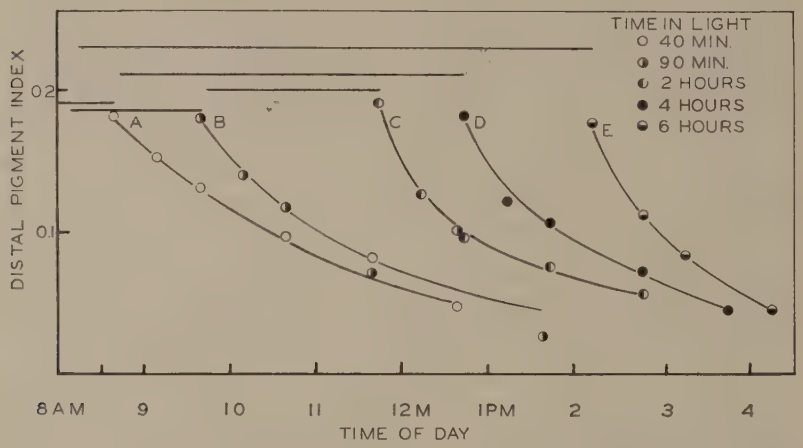

Fig. 1 The effect of duration of light period on dark-adaptation in *Palaemonetes*. Curve A represents dark-adaptation after 40 minutes in the light; curve B, after 90 minutes; curve C after 2 hours; curve D, after 4 hours; curve E, after 6 hours.

son of the curves reveals a general increase in rate of dark-adaptation as the duration of light exposure increases. In accordance with the two-hormone hypothesis of distal pigment regulation, this increase in rate is taken to indicate an increase in the amount of available dark-adapting hormone.

The results recorded in figure 1 suggest that there can be an increase in the amount of dark-adapting hormone under conditions of illumination. Brown, Hines, and Fingerman ('52) recorded an increase in amount in darkness. It seems unlikely that both conditions would constitute a stimulus to

production of the hormone. Although the rate of dark-adaptation is conspicuously greater after 6 hours of illumination (curve E, fig. 1) than after 40 minutes (curve A, fig. 1) a detailed comparison of the curves shows only a very slow increase in rate of dark-adaptation with increase in duration of the light period. Thus curves A and B indicate closely similar rates in spite of the fact that the light period has been doubled in the second test. But in proceeding from curves A through E it is clearly evident that progressively shorter times are required to move this distal pigment from the light-adapted state to some arbitrary index, as for example 0.06. It will be observed, however, that these experiments were performed in such a manner that each successive curve, A through E, represents dark-adaptation at a later time of day as well as following increasing periods of illumination. There are, therefore, two variables whose effects cannot be distinguished in these experiments: time of day, and duration of the light period.

2. Influence of time of day on rate of dark-adaptation. A group of animals was dark-adapted for 10 hours from 10 P.M. to 8 A.M. These dark-adapted animals were then separated into 5 pans of 10 animals each and exposed to illumination of 250 ft. c. for one and one-half hours and then returned to the dark. One pan was removed from the dark and the average condition of the retinal pigments determined at each of the following times: 30, 60, 120, 180, and 240 minutes after being placed in the dark. Another lot of animals which had been dark-adapted for 10 hours from 8 A.M. to 6 P.M. were exposed in a similar manner to illumination of 250 ft. c. for one and one-half hours and their rate of dark-adaptation followed. It was necessary to examine groups from this latter series only until 90 minutes as they were completely dark-adapted at that time.

The results from this experiment are presented in figure 2. It is immediately clear that a great difference in rates exists. The rate for the animals dark-adapting at 7:30 P.M. (curve A) approaches the maximum rates observed, whereas

that for animals dark-adapting at 9:30 A.M. (curve B) is much slower and the animals have not completed the process even after 4 hours in the dark. Both groups have been exposed to 10 hours of darkness followed by one and one-half hours of light. It seems, therefore, that the time of day is an important factor in determining the ability of *Palaemonetes* to dark-adapt. If the rate of dark-adaptation is a function of the concentration of a dark-adapting hormone then the amount of dark-adapting hormone available must vary throughout the day under these conditions.

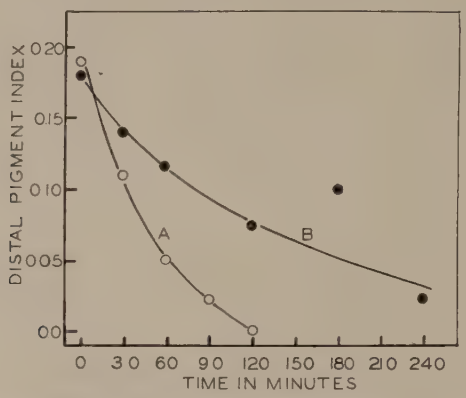

Fig. 2 The effect of time of day on dark-adaptation in *Palaemonetes*. Curve A represents animals dark-adapting at 7:30 P.M.; curve B, at 9:30 A.M. Both groups of animals had been in the dark for 10 hours and then illuminated for one and one-half hours.

3. Response to a one-minute light stimulus throughout the diurnal cycle. The test stimulus for this experiment was exposure to one minute of light at an illumination of 250 ft. c. Animals were placed in the dark in the evening. At various times of day during the succeeding two days these dark-adapted animals were tested by exposing 5 containers, each containing 10 animals, to light at 250 ft. c. for one minute. The containers were covered at the end of one minute and returned to the darkroom. The state of the distal pigment of one group of 10 animals was determined 30 minutes after the end of the exposure to light, that of other groups of

10 animals at 60, 120, and 240 minutes after exposure to light. Animals were prepared and tested in this manner on three different occasions and a total of 13 test exposures were performed. In three cases it will be noted that animals were tested at about the same hour on different days.

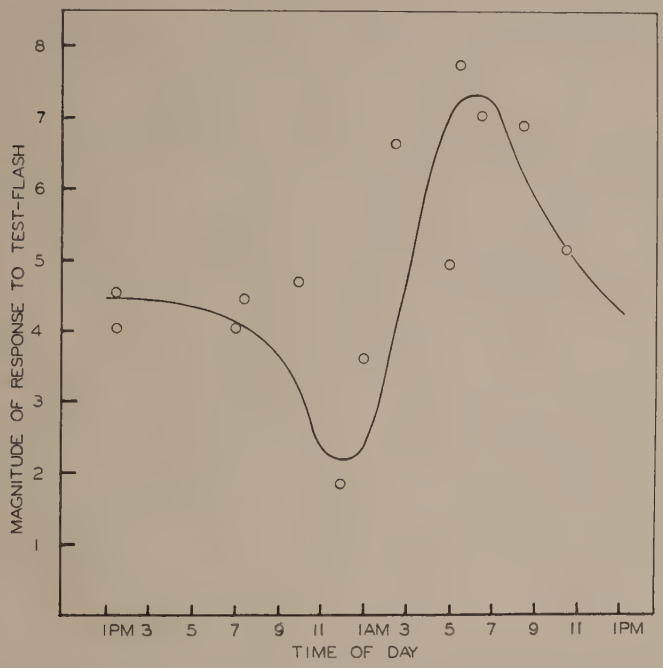

Fig. 3 Curve showing relative magnitude of response of dark-adapted *Palaemonetes* to exposure to one minute of light at 250 ft. c. at various times of day. (See text for explanation of figure.)

The results of this series of experiments are presented in figure 3. In this figure the numerical values representing total response for each time tested, that is, the sum of the average distal pigment indices for the 4 times at which the state of the pigment was determined, are plotted as a function of the time of day. The time coordinate represents a single 24 hour cycle.

Examination of the graph reveals that response is at an intermediate level at about 1:30 P.M. and remains so until

about 7 P.M. Shortly thereafter, an abrupt decrease occurs so that a minimum response is observed at midnight. This is followed by a steep increase until by 5 A.M. the maximum response is obtained. There then follows a gradual decrease until about 9 A.M. and then a more rapid decrease until at

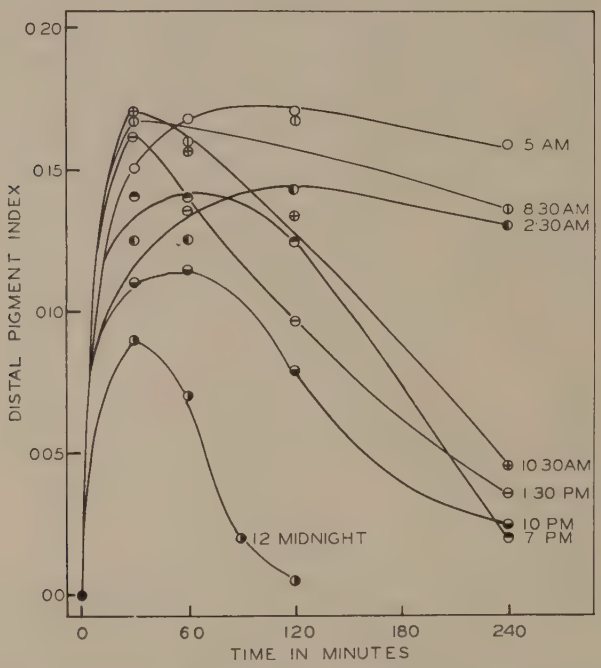

Fig. 4 Responses of dark-adapted *Palaemonetes* to exposure to one minute of light at 250 ft. c. at various times of day.

11 A.M. values approaching those seen at 1:30 P.M. are obtained. There is, therefore, a clearly observable diurnal rhythm of response to the test stimulus.

In figure 4 are seen sample data obtained from experiments performed at different times in the diurnal cycle at 12 P.M., 2:30 A.M., 5 A.M., 8:30 A.M., 10:30 A.M., 1:30 P.M., 7 P.M., and 10 P.M. The curve in figure 4 illustrating the response obtained when the test was performed at 12 midnight shows that at this time there is a relatively low amplitude

of response and a rapid rate of re-dark-adaptation. At 5 A.M., the other extreme, the amplitude of the response is great and the rate of return to the dark-adapted condition is extremely slow. If maximal rates of dark-adaptation are attributable to the presence of a dark-adapting hormone, this hormone must be available in maximal amounts only for a relatively brief period near midnight in these animals which are kept in constant darkness. Between these 5 A.M. and 12 midnight values intermediate amounts are present.

Since the values plotted in figure 3 represent relative degrees of response and a low value for the total response is correlated with a rapid rate of re-dark-adaptation, these values are inversely related to the relative amounts of dark-adapting hormone available at the indicated times. A curve indicating the relative amounts of dark-adapting hormone available throughout the 24 hour cycle would show a maximum at or near midnight and a minimum near 5 A.M.

If the extent of light-adaptation in any test represented exclusively a measure of the amount of light-adapting hormone available then there would also be a definite diurnal rhythm in availability of this hormone. But an examination of figure 4 clearly shows that the differences among the measurements are chiefly due to differences in the rates of re-dark-adaptation following the maximal light-adaptation in response to the flash. At some times of day the return towards the dark-adapted state begins quickly and proceeds rapidly. At other times, it starts slowly and gradually accelerates. From 2:30 to 5:00 A.M., there is no great tendency to return to dark-adaptation even by the end of 4 hours. It is very clear therefore that there are enormous differences in the capacity to produce dark-adapting hormone in darkness at the different times of day. It seems reasonable to presume that, since the dark- and light-adapting hormones have antagonistic actions, at least some of the difference in amount of light-adaptational response to the one-minute flash is due to differences in quantity of the dark-adapting antagonist present. It is not possible at this time to conclude, however, that this is the total

explanation and that a rhythm in immediate availability of light-adapting hormone is absent.

4. Effect of constant illumination on rate of dark-adaptation. Animals were placed upon a white background at a light intensity of 250 ft. c. at 4 P.M. This served as a stock supply from which animals were tested for their ability to dark-adapt at various times. At any one test 4 groups of 10 animals each were removed from the stock supply and placed in the dark. The state of the distal retinal pigment was observed in 10 animals by the usual method at the time the change

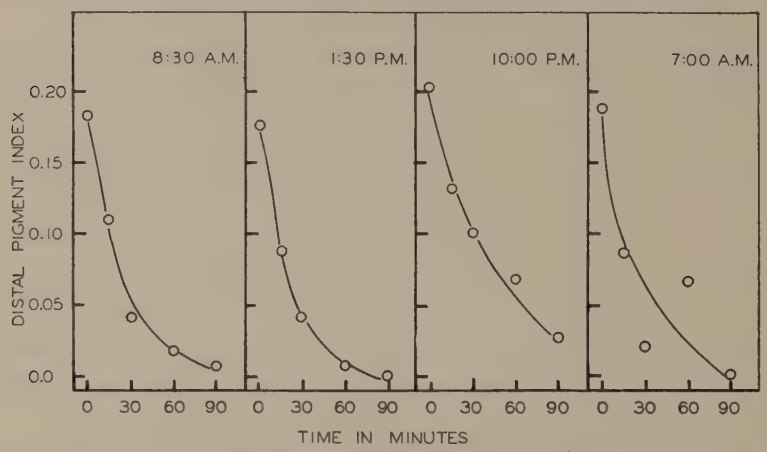

Fig. 5 Dark-adaptation at various times of day of *Palaemonetes* previously maintained in constant illumination of 250 ft. c.

was made. The state of the retinal pigments was similarly determined for one group at each of the following times after the animals were placed in the dark: 15, 30, 60, and 90 minutes. In this manner the rate of dark-adaptation was determined for each of the following times of day: 7 A.M., 8:30 A.M., 1:30 P.M., and 10 P.M.

The results of this experiment are shown in figure 5. It can be seen that for three of the times at which tests were made the rates are practically identical. At the 4th time (10 P.M.) the rate is somewhat less than that observed at the other three times. That this low rate is not the result

of injury from prolonged exposure to light is shown by the fact that 10 P.M. was the first time at which dark-adaptation was tested. The animals in this test had been exposed to light for only 6 hours while those tested at 8:30 A.M. had been in light for about 16 hours. Whether the slow rate observed at 10 P.M. is characteristic for that particular group of animals or is in some way related to the illumination it is clear that under conditions of constant illumination an inhibition of the previously observed rhythm in availability of dark-adapting principle occurs.

A second experiment was performed testing the ability of animals to dark-adapt from conditions of constant illumination. In this experiment the stock animals were maintained in a large laboratory aquarium with a surface illumination of 250 ft. c. The illumination to which these animals were exposed was therefore considerably less than that used in the previous experiment. Throughout a 5-day period the ability to dark-adapt was tested at the following times: 1 P.M., 1:30 P.M., 4 P.M., 5 P.M. (two tests), 9 P.M., 12 midnight, 5 A.M., 9 A.M. (two tests), and 10 A.M. The average state of the retinal pigments was determined when the animals were to be placed in the dark and after 30, 60, and 90 minutes in the dark.

A comparison of the rates of dark-adaptation failed to reveal any differences throughout the day. It was concluded that under conditions of constant illumination no diurnal rhythm in rate of dark-adaptation could be demonstrated and hence no diurnal variation in the availability in dark-adapting hormone in response to a light-to-dark change.

5. Effect of constant low illumination on the position of distal retinal pigments. On the basis of the results of the preceding experiments it was postulated that if in *Palaemonetes* there was a significant diurnal rhythm in availability of dark-adapting hormone, but little or no similar rhythm for the light-adapting hormone, then there could well be a visible pigmentary rhythm under conditions where the state of adaptation of the pigment was in good measure dependent upon

the presence of dark-adapting principle. Since the pigment remained in the fully dark-adapted state at all times of day in constant darkness it was evident that at least the minimal amount of hormone for maximal dark-adaptation was produced at all times, and therefore this did not provide a satisfactory condition. Instead, a constant and low illumination of the eye was used.

A group of 10 animals was placed upon a black background and exposed to constant illumination of 10 ft. c. intensity for two days. This provided an effective illumination for the eye 0.014 times that provided when a white background was em-

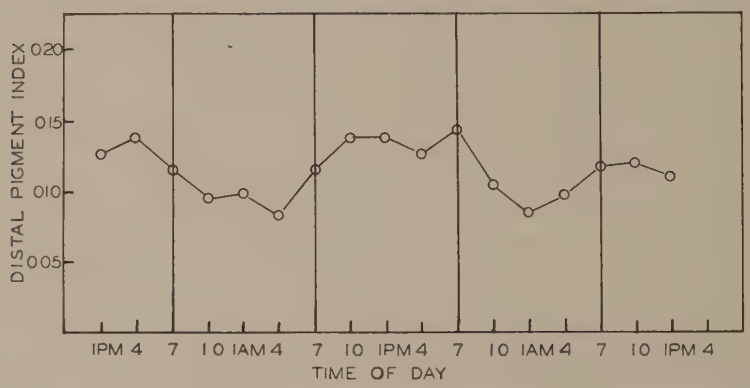

Fig. 6 Diurnal rhythm of the distal retinal pigment of *Palaemonetes* maintained on a black background at an illumination of 10 ft. c.

ployed (Sandeen and Brown, '52). During this period the state of the distal retinal pigment was determined each day at the following times: 1 P.M., 4 P.M., 7 P.M., 10 P.M., 1 A.M., 4 A.M., 7 A.M., and 10 A.M.

The results of this experiment are presented in figure 6. It can be seen that the pigment is in a more dark-adapted position at the hours of 10 P.M., 1 A.M., and 4 A.M., than at other times throughout the 24 hour cycle. Thus a diurnal rhythm of the distal retinal pigment is evident. Under these conditions of low light intensity the retinal pigment is never in a completely light-adapted condition but is more nearly light-adapted during the daytime hours than at night. The excur-

sion of the rhythm as indicated by the difference between the maximal index and the minimal index for each day is approximately 38% of the possible range.

DISCUSSION

The results described here indicate that for three differing conditions of illumination — constant darkness, constant illumination, and normal day-night conditions — *Palaemonetes* exhibits three different patterns of physiological activity of the distal pigment regulating mechanism.

In constant darkness a persistent diurnal rhythm of response to a one-minute light flash is observed and this rhythm has been interpreted chiefly in terms of variations in the amount of a dark-adapting hormone present. The pattern of this rhythm is such that from about 7 P.M. to midnight the amount of dark-adapting hormone present becomes maximal. During this time the response of the distal pigment to the light flash becomes small and the rate of re-dark-adaptation becomes rapid. Shortly after midnight a reversal occurs so that by 5 A.M. very small amounts of dark-adapting hormone are present. Throughout much of the day intermediate levels of dark-adapting hormone are available. It is quite possible, though not capable of proof from these experiments, that a reciprocal diurnal alteration in availability of a light-adapting hormone for response to the light flash also occurs.

In constant illumination the dark-adapting hormone appears to be available at all times in sufficient amounts to permit approximately maximal rates of dark-adaptation when the animals are transferred to the dark. There is some evidence that this involves an accumulated store of hormone. These animals appear to remain continuously in the phase of maximal availability of the dark-adapting hormone for response to light-to-dark change. There is as yet no evidence that animals in constant darkness accumulate comparable stores, but rather appear to exhibit a diurnal rhythm in the rate of productioin of the dark-adapting hormone which may be liberated into the blood in darkness as fast as it is pro-

duced. It is possible that it is accumulated in glandular sources only under conditions of high illumination.

In constant illumination of sufficiently low intensity, an observable diurnal rhythm in the position of the distal retinal pigment exists. This rhythm is characterized by the pigment becoming more dark-adapted during the hours from 10 P.M. through 4 A.M. A change in the position of the retinal pigment toward the position characteristic of dark-adaptation could presumably be brought about either by an increase in the amount of dark-adapting hormone in the blood or by a decrease in the amount of light-adapting hormone. Since the only distal pigment hormone which has been clearly demonstrated to exhibit a persistent diurnal rhythm in its rate of production is the dark-adapting one, it is assumed that it is the one chiefly responsible for the rhythm. Were there a comparable rhythm for the light-adapting hormone, one would, especially in view of the dominance of this principle, perhaps have expected to observe a distal pigment rhythm in constant darkness. This, however, is not the case. The observed absence of a distal pigment rhythm in *Palaemonetes* in constant bright light may be due to powerful regulation to intensity or to the lack of a rhythm in the light-adapting hormone which would be the principal one operative under these conditions of illumination.

Palaemonetes under normal day-night conditions present a still different pattern of activity from those already described. Animals which are placed in the dark in the evening and tested by exposure to one minute of light at 250 ft. c. during that night exhibit the typical nocturnal phases of the diurnal rhythm found in constant darkness. Presumably animals under normal conditions are exposed to darkness at night. It therefore follows that *Palaemonetes* normally undergoes a marked decrease in ability to dark-adapt and a marked increase in ability to light-adapt shortly after midnight. But animals which have been exposed to dawn and the normal increasing light intensities associated with the change from night to day are capable of dark-adapting at maximal rates

(Brown, Fingerman, and Hines, '52), only 60 to 90 minutes being required for the total change. The ability of the animals in the "morning phase" of the rhythm in constant darkness to dark-adapt, on the other hand, may be seen in curve A of figure 1. There is evidence that the observed difference is the result of the early morning period of low intensity illumination to which the normal animals have been exposed. The diurnal pattern of hormone production under normal day-night conditions then would consist of a marked nocturnal decrease in dark-adapting hormone shortly after midnight followed by a rapid increase in availability in response to the dawn illumination and a high level of availability would then be normally maintained, probably through storage, throughout the day. Throughout the daytime hours in bright daylight there is apparently only a low productive capacity for dark-adapting hormone.

Although animals in constant bright illumination were shown to be continuously in a state of low production of the dark-adapting hormone during the daytime hours, illumination at high intensities does not induce this phase directly. This can be seen from the results presented in figure 1. Even after 6 hours of illumination at 250 ft. c. the rate of dark-adaptation of these animals was similar to that of animals tested from constant darkness at the same time of day. Moreover, the period of most rapid increase in rate of dark-adaptation occurs at a time when a similar rapid increase in ability to dark-adapt was noted for animals maintained in constant darkness. Thus there appears to be little effect of bright illumination during this phase of the cycle.

In the investigation of the effect of constant illumination animals which had undergone normal night-to-day changes were used. Animals were selected from the laboratory aquarium during the daytime and placed in constant illumination. These animals were capable of maximal rates of dark-adaptation at the beginning of the experiment and remained so throughout.

SUMMARY

1. *Palaemonetes vulgaris*, in constant darkness, exhibit a persistent diurnal rhythm in their capacity to produce dark-adapting hormone for the distal retinal pigment.

2. In constant darkness, this capacity is maximal at about midnight and minimal at about 5 A.M.

3. Under conditions of continuous bright illumination, the prawns are able to dark-adapt at equal and maximal rate at any time during the diurnal cycle, probably due to possession of a stored accumulation of dark-adapting hormone.

4. The persistent rhythm in the production of dark-adapting hormone normally has superimposed upon it a direct induction by the daily light cycle. Low intensity of dawn illumination results in rapid production of dark-adapting hormone at a time when this is not demanded by the endogenous rythmic regulating mechanism.

5. A distinct rhythm of the distal pigment of *Palaemonetes* is evident under constant conditions only when intensity of illumination is of such magnitude that the distal pigment is in an intermediate state of light adaptation.

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DIFFERENTIAL PRODUCTION OF TWO RETINAL PIGMENT HORMONES IN PALAEMONETES BY LIGHT FLASHES

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TEN FIGURES

INTRODUCTION

Previous investigations (Webb and Brown, '52; Brown, Fingerman, and Hines, '52) of the control of the state of the distal retinal pigment of the prawn, *Palaemonetes,* have led to the postulation of the existence of a dark-adapting hormone in addition to a light-adapting hormone (Kleinholz, '36; Welsh, '39). The dark-adapting hormone was first postulated to explain the difference between the rate of re-dark-adaptation of prawns kept in darkness overnight when returned to darkness following a brief 250 ft. c. light exposure early in the morning and the rate of dark-adaptation of prawns which had earlier in the same day been exposed to the normal stimulus of dawn illumination before being dark-adapted and then subjected to the brief 250 ft. c. exposure. Brown, Hines, and Fingerman ('52) later demonstrated that both the eyestalks and central nervous organs of *Palaemonetes* possess both the light-adapting and dark-adapting hormones, while the tritocerebral commissure possesses the dark-adapting hormone but no light-adapting factor.

[1] This investigation was supported in part by a research grant from the Graduate School of Northwestern University.

When one compares the responses of the distal retinal pigment of different animals to an abrupt change from bright light to darkness one finds that the rate of the process of dark-adaptation is influenced in some manner by the previous light history of the animals and by the stage in the diurnal physiological cycle. Prawns which have been permitted to light-adapt in response to the normal increasing illumination of the dawn are able any time thereafter during the daytime hours to dark-adapt at a maximum rate in response to darkness, the process starting rapidly and going to completion usually in 60 to 90 minutes. This occurs at 6 to 9 A.M. in the diurnal physiological cycle just as readily as at later times in the day. If, on the other hand, prawns have been kept in continuing darkness through the early morning hours they will, in response to a period of artificial illumination at 250 ft. c. of one or more minutes' duration, light-adapt more or less completely, but then can re-dark-adapt only very slowly following their return to complete darkness. Under these last conditions the process requires many hours; in fact, they do not become completely dark-adapted until evening.

The foregoing phenomenon was described by Brown, Fingerman, and Hines ('52) when they described the rates of dark-adaptation of the distal pigment of the eye in (A) *Palaemonctes* which have been subjected to normal dawn increase in illumination and were at about 10 ft. c. at the time of transfer to darkness, (B) animals which have been subjected to normal dawn increase in illumination but which were at about 1 ft. c. when transferred to darkness, and (C) animals which were retained through the early morning hours in darkness, but which at the time the preceding two groups of animals were placed in darkness, were subjected to a 1-minute flash of light at 250 ft. c. and then returned to darkness.

It was found that the rates of dark-adaptation were essentially the same in the first two groups, and strikingly different from that of the third one. In all three groups the change from light to darkness was made at the same time in the diurnal cycle, namely between 5 and 5:30 A.M. The only dif-

ference in the immediate history of the three groups was the character of light stimulation preceding the transfer to darkness.

Furthermore, modifications of this type of experiment (Webb and Brown, '52) gave good reason to believe that a period of illumination at 250 ft. c., the equivalent in length to that of the previous light exposure of the animals subjected to the normal dawn illumination changes, would, as in the case of the 1-minute flash, be followed upon transfer to darkness by the very slow dark-adaptation over many hours. These facts suggested rather strongly that some aspect of the gradually increasing dawn illumination was responsible for providing the animals with the later ability to re-dark-adapt rapidly.

The following experiments were designed in order to discover what aspect of dawn-illumination was responsible for providing the prawns with the capacity to dark-adapt rapidly later and what were the characteristics of action of this factor.

MATERIALS AND METHODS

All the *Palaemonetes vulgaris* used in the following experiments were freshly collected for each experiment in order to minimize the possibility that any alteration in the phases of their persistent daily rhythm would occur. The animals were collected from sea weeds in the Eel Pond near the Marine Biological Laboratory.

Quantitative measurements of the state of the distal retinal pigment of the prawns were made with the aid of a binocular dissecting microscope possessing an ocular micrometer. At a magnification of about 40 × it was possible to measure very rapidly the width of the clear area in the peripheral region of the eye which was due to the proximal migration of the distal pigment in the course of light adaptation. Since the animals which were used in the experiments exhibited a wide range of sizes it was judged desirable to express this measurement as a ratio of some measurement of eye-size. For this

last, the distance from the distal extremity of the eyestalk to the proximal edge of the dorsal "ocellus" lying at the base of the eye proper was used. This ratio will be referred to as the distal pigment index. Although this index possibly varies slightly with the size of the prawn, it varies substantially less than the width of the area for any given degree of light adaptation by itself.

In each of the determinations of the distal pigment index 10 animals were examined and the average index for the group was obtained. In all instances when it was necessary to follow in time changes which occurred in the pigments in response to a given experimental condition as many lots of 10 animals were prepared and participated in the experiment as there were eventual determinations to be made. The animals were kept in white- or black-enamelled pans. Each determination involved utilizing one of the pans of animals as a sample and then discarding it. Consistency in the results of numerous experiments in which this technique has been used has justified the method completely.

Inasmuch as previous experiments have clearly indicated that the character of the distal pigment response is subject to alterations by many factors, including a persistent daily rhythm, all of the experiments in which detailed comparisons have been made among numerous experimental series have been performed at approximately the same time each day. The particular time of day at which the specific characteristics of the diurnal cycle were most favorable for most of the experiments to be described in the present report was early in the morning. Consequently, the great majority of the experiments were begun at between 5 and 7 A.M. and the prawns which were used for these experiments had been in continuous darkness from the preceding day. These animals had not been subjected to the modifying influences on the basic diurnally rhythmic mechanism which are effected as the animals are subjected to the normal gradual increase in illumination of the dawn.

EXPERIMENTS AND RESULTS

The dawn increase in illumination was considered to be a conditioning stimulus which in some manner altered the character of the later response to the test flash which consisted of a 1-minute exposure to light at 250 ft. c. Prawns which had been in constant darkness since the preceding day were given various conditioning light stimuli preceding the 1-minute flash. It was first discovered that removal of the animals from darkness in the morning to an illumination of 0.1 ft. c. where they were left for an hour preceding the 1-minute test flash, would result in re-dark-adaptation following the flash at a rate quite comparable to that observed in ones subjected to normal dawn illumination. Furthermore, such a conditioning stimulus at 0.014 ft. c. would have a similar influence whether the duration was 30 minutes or 75 minutes. It, therefore, appeared quite definite that conditioning stimuli within this range of intensities, if allowed to operate for at least 30 minutes, would induce some modification within the animals comparable to that induced by the normal increasing dawn illumination in permitting subsequent rapid dark-adaptation.

Additional exploratory experiments were performed varying the intensity of the conditioning stimulus, its duration and the time of its action relative to the test flash. In one experimental series three types of conditioning stimuli were given: the 250 ft. c., 1-minute flash was preceded by (1) a 1-minute, 0.014 ft. c. flash followed by 29 minutes in darkness; (2) a 1-minute, 2 ft. c. flash followed by 29 minutes in darkness; and (3) a 1-minute, 10 ft. c. flash followed by 29 minutes in darkness. These experiments were all started between 6 and 6:15 A.M. In figure 1 are plotted, along with later results, the results which show the subsequent rates of dark-adaptation of these three groups of animals together with the rates on a control group which had had no conditioning treatment.

Another exploratory series involved 6 types of conditioning stimuli as follows: (1) 0.014 ft. c. for 30 minutes followed at once by the 1-minute, 250 ft. c. flash; (2) 50 ft. c. for 1

minute, then 29 minutes in dark, followed by the 1-minute, 250 ft. c. flash; (3) 2 ft. c. for 1 hour followed at once by the 1-minute, 250 ft. c. flash; (4) 1 ft. c. for 20 minutes followed at once by the 1-minute, 250 ft. c. flash; (5) 1 ft. c.

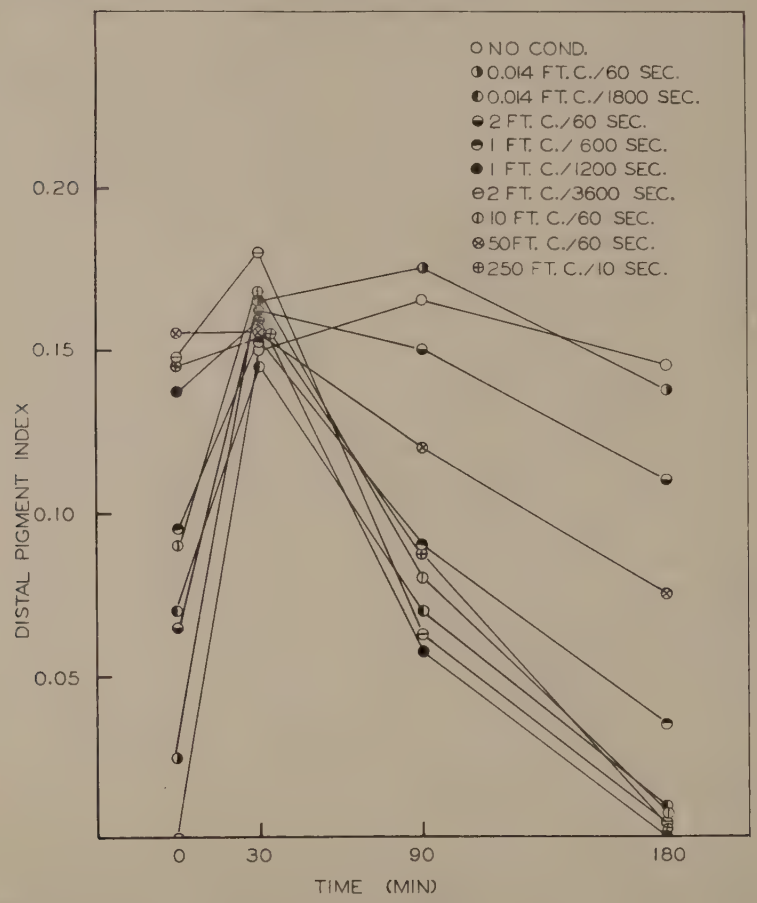

Fig. 1 Responses of overnight, dark-adapted prawns to a 1-minute, 250 ft. c. test flash, following various conditioning light stimuli.

for 10 minutes followed at once by the 1-minute, 250 ft. c. flash; (6) 250 ft. c. for 10 seconds, then 30 minutes in darkness followed by the 1-minute, 250 ft. c. flash. These experiments were all commenced between 7 and 7:15 A.M. Figure

1 graphically depicts the rates of dark-adaptation of these groups of animals as they remained in darknese after the experimental treatments.

It will be readily observed from the preceding two series of experiments that 10 ft. c. illumination for 1 minute followed by 20 minutes in darkness preceding the test flash of 250 ft. c. provided the prawns with a capacity to dark-adapt in darkness thereafter at a rate quite comparable to that seen in animals which had been exposed to the natural increasing dawn illumination. Two hundred and fifty ft. c. for 10 seconds followed by 30 minutes in darkness appeared to be equally effective in this capacity. On the other hand, neither 0.014 ft. c. nor even 1 ft. c. for 1 minute followed by 29 minutes in darkness had other than a relatively small influence in this regard. The other types of conditioning stimuli gave results which were intermediate respecting this capacity.

It is quite evident from the preceding experiments that both intensity and duration of the conditioning light stimuli are factors in the determination of the subsequent ability of the animals to dark-adapt in darkness. Also, the time intervening between such conditioning stimuli and the time of the test flash appear to be of significance in this respect. To gain some further insight into the contributions of each of these factors, and to some extent the interrelationships of these, 4 more series of experiments were performed.

Two hundred and fifty ft. c. as the conditioning illumination. In the first of these series the intensity of light was held constant at 250 ft. c. for the conditioning stimulus. The durations of the light period were: 1 second, 2 seconds, 5 seconds, 20 seconds, 40 seconds, 1 minute, 5 minutes, 15 minutes, and 30 minutes. (The last value was repeated twice.) Following the light period each group (except the 30 minute one) was placed in darkness for a period of such length as to yield a total time of 30 minutes from the beginning of the conditioning. All were then given the 1-minute, 250 ft. c. test stimulus and then returned to darkness. Figure 2 illustrates the different rates of dark-adaptation seen among the

different groups. It is quite evident that the maximum facilitation of subsequent dark-adaptation under the conditions of this experiment occurs as a result of a 5- to 10-second exposure to the conditioning light. Longer or shorter exposures are less effective.

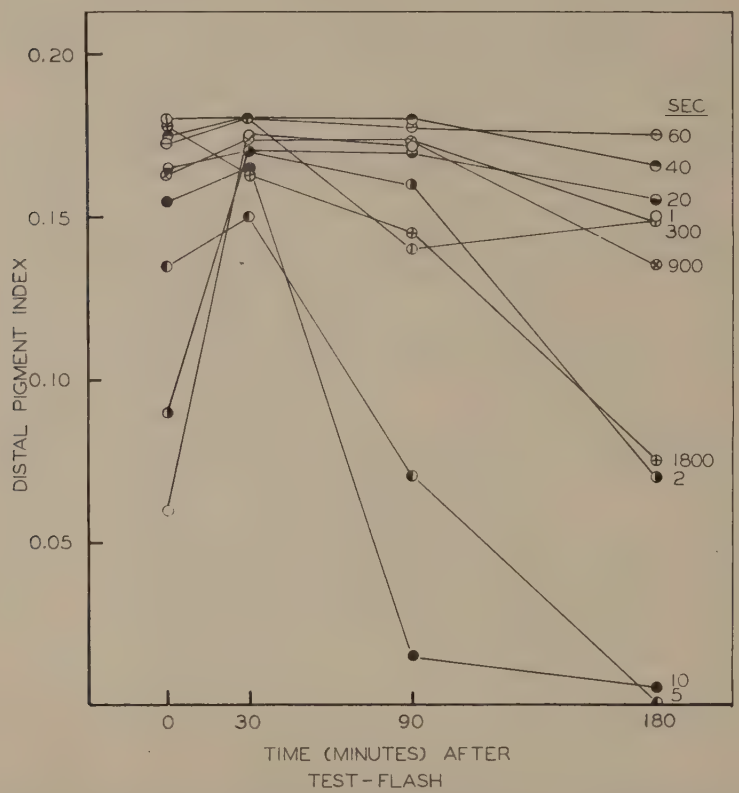

Fig. 2 Responses of overnight, dark-adapted prawns to a 1-minute, 250 ft. c. test flash, a half hour after the initiation of 250 ft. c. conditioning stimuli of various durations.

Fifty ft. c. as the conditioning illumination. In the second series, the intensity of the conditioning light stimulus was held constant at 50 ft. c. The durations of the light periods for the different groups in this series were: 5 seconds, 15 seconds, 30 seconds, 1 minute, 5 minutes, and 30 minutes.

(The last value was repeated once.) Following the light period each group except the 30-minute ones was placed in darkness for a period to bring the total of light and dark periods to 30 minutes. All were then given the 1-minute, 250 ft. c. test stimulus and returned to darkness. Figure 3 shows the

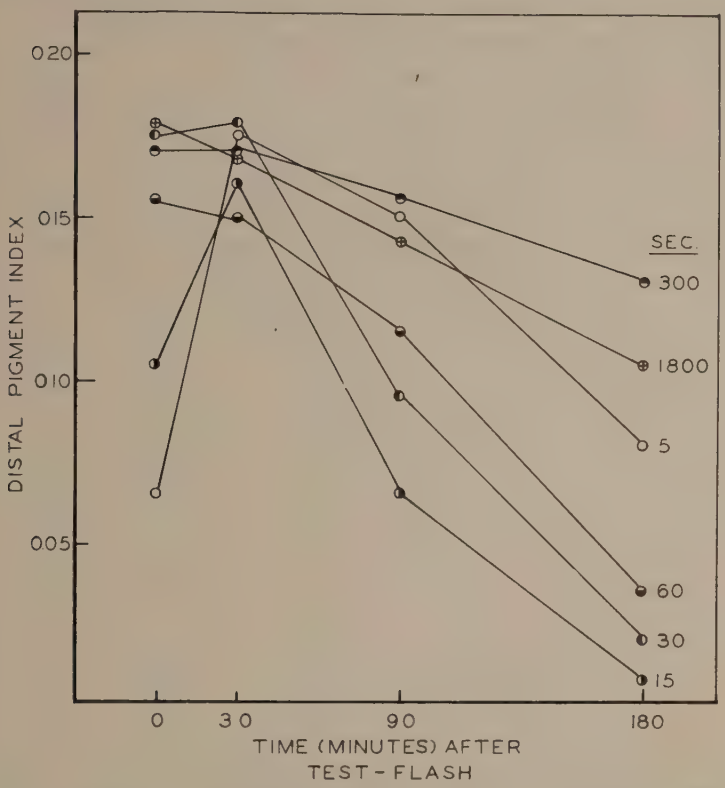

Fig. 3 Responses of overnight, dark-adapted prawns to a 1-minute, 250 ft. c. test flash, a half hour after the initiation of 50 ft. c. conditioning stimuli of various durations.

rates of dark-adaptation of these groups following this treatment. Here it is seen that the 15-second conditioning light stimulus provides the maximum facilitation of the subsequent dark-adaptation, the effect of longer and shorter light periods being less.

Ten ft. c. as the conditioning illumination. The intensity of the conditioning light stimulus was held at 10 ft. c. for the third series. Here the durations of the light periods for the different groups were: 15 seconds, 30 seconds, one minute, 3 minutes, 5 minutes, and 30 minutes. (The 3-minute

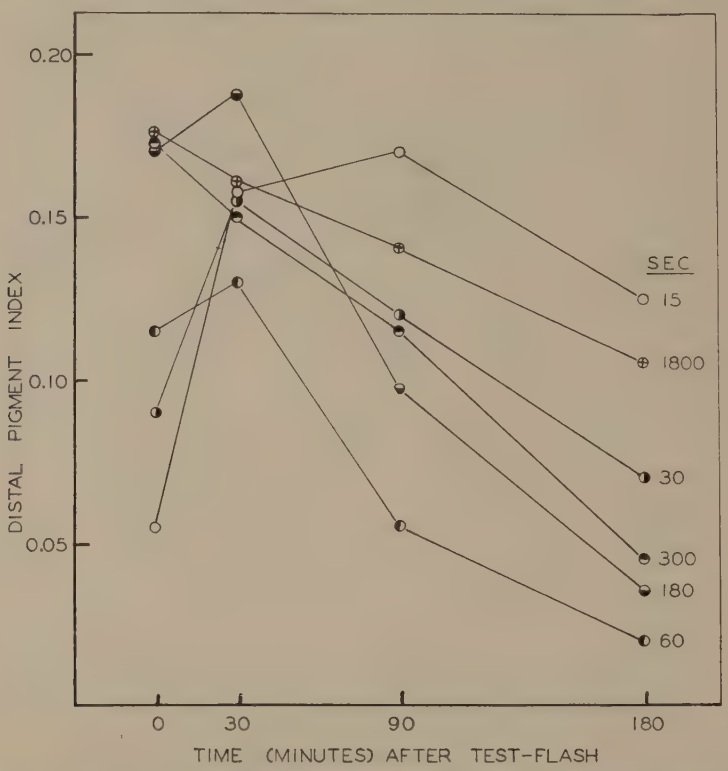

Fig. 4 Responses of overnight, dark-adapted prawns to a 1-minute, 250 ft. c. test flash, a half hour after the initiation of 10 ft. c. conditioning stimuli of various durations.

and the 30-minute values were each repeated once.) Each group was placed in darkness for a period sufficient to make the sum of the light and dark periods 30 minutes. At the end of this time all groups were given the 1-minute, 250 ft. c. test stimulus and returned to darkness. Figure 4 graphically depicts the rates of dark-adaptation of each of these groups

following the return to darkness. At this intensity of conditioning stimulus the duration for maximum facilitation is seen to be 1 minute, both shorter and longer times being less effective.

Two ft. c. as the conditioning illumination. In the last of these series the illumination for the conditioning stimulus was maintained at 2 ft. c. The durations of the light periods in this series were: 1 minute, 2 minutes, 5 minutes, 10 minutes, and 30 minutes. Each group was then placed in darkness for a period long enough to make the total time of light and darkness of the conditioning period 30 minutes, at the end of which time each was given the 1-minute, 250 ft. c. test stimulus. Figure 5 shows the rates of dark-adaptation of the various groups subsequently in darkness. Unlike in the preceding three series, here there is not so clearly an optimum duration of the conditioning stimulus as in the other series. Possibly one occurs at 300 seconds. In a general way, the longer the period of the conditioning light up to the maximum length used (30 minutes) the more the facilitation of the subsequent process of dark-adaptation. Furthermore, for the shortest conditioning light stimulus used, 1 minute, facilitation is already considerable.

General intensity-duration relationship. In order to compare more readily the relative rates of dark-adaptation following the various intensities and duration of the conditioning stimulus, figure 6 has been prepared. In this figure, for convenience of plotting, the abscissal value indicates the common logarithm of the duration of the conditioning stimulus in seconds. The ordinate gives the sum of the average distal pigment indices at 30 minutes, 90 minutes, and 180 minutes, following the final return of the prawns to darkness after the 1-minute test stimulus. This sum was considered to provide a good measure, for comparative purposes, of the reciprocal of the rate of re-dark-adaptation. The larger this value, the lower the rate of dark-adaptation; the smaller this value, the higher the rate. All the curves are drawn to converge at the left at about 0.5 since this was found to be the

approximate value for this time of day for those animals which had received no conditioning light prior to a test flash. On this graph are included not only all the data obtained from the 4 series described earlier, but also comparable additional data gleaned from the initial exploratory experi-

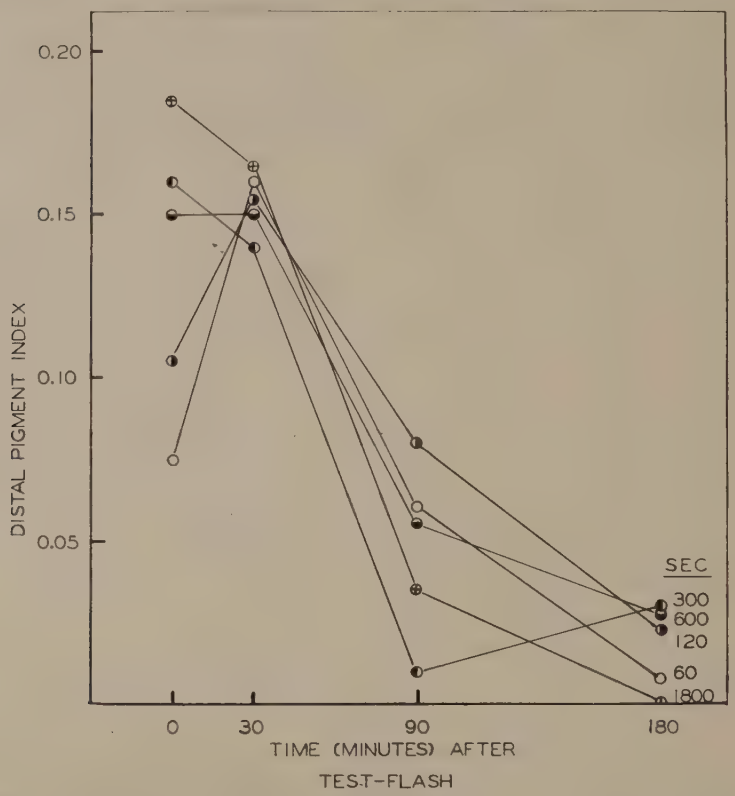

Fig. 5 Responses of overnight, dark-adapted prawns to a 1-minute, 250 ft. c. test flash, a half hour after the initiation of 2 ft. c. conditioning stimuli of various durations.

ments. Also included are two points for a conditioning stimulus of 0.014 ft. c. which enables one to include the probable form of a 5th curve in the series. This 5th curve fits into an entirely consistent picture with the remaining 4. It is interesting for comparative purposes that an ordinate value of 0.085

indicates a comparable measure of the rate of dark-adaptation of prawns which have been allowed to light adapt in the normal increasing dawn illumination and then, at the time of day of these experiments, are simply placed in darkness.

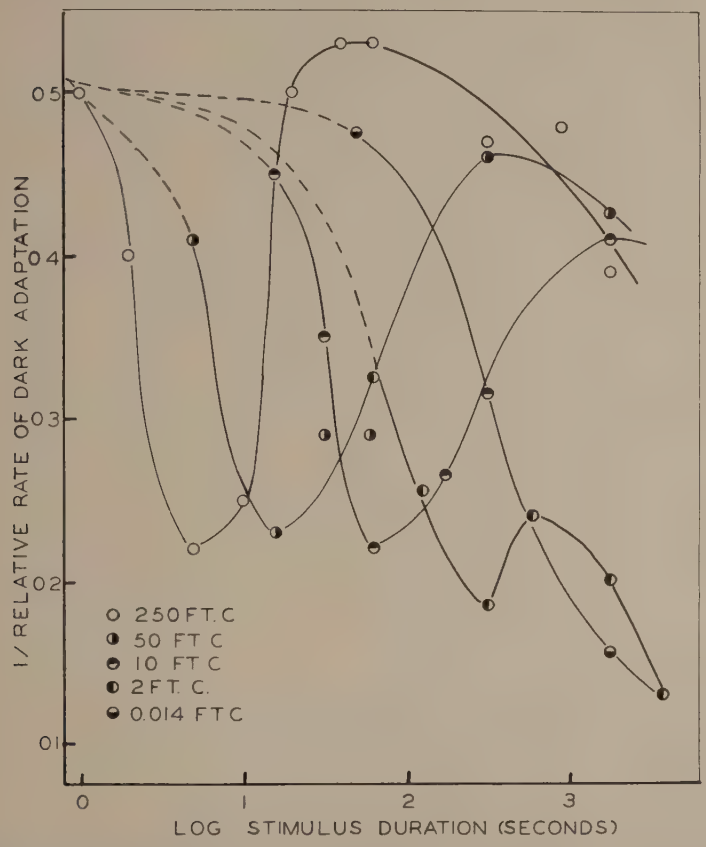

Fig. 6 The relationship between log conditioning stimulus duration and the reciprocal of dark-adapting facilitation for 5 intensities of illumination.

It is readily evident from figure 6 that at illuminations from 250 ft. c. down through 10 ft. c. the duration of the conditioning light stimulus for optimum facilitation of the subsequent dark-adapting process increases with decreasing intensity, and that for still lower illuminations the conditioning

period must be progressively longer to induce any given amount of such facilitation. Furthermore, at illuminations above some value lying between 0.014 and 10 ft. c., an increase in duration of the conditioning light stimulus beyond some optimum time actually inhibits the facilitation to a greater or lesser degree. But after a still longer period of time there is a secondary facilitation which occurs much more gradually than the initial one. This inhibition is possibly absent at 2 ft. c. and probably absent at lower intensities. When secondary inhibition and re-facilitation occur, the duration of the conditioning stimulus for onset of these is an inverse function of the intensity of the illumination, and the degree of inhibition is a direct function. There is a gradual drop-off in inhibitory action from 250 ft. c. through 10 ft. c., and then a precipitous drop between 10 and 2 ft. c.

Rate of generation of dark-adapting capacity. Since it is now quite apparent that in a half hour of darkness following a 5-second, 250 ft. c. flash the prawns develop some physiological state which enables them to dark-adapt rapidly thereafter, and this change in darkness fails to occur following a 1-minute flash, it would follow that the conditioning light stimulus had left the animal in a physiological state such that one or another process would persist in subsequent darkness. It became of interest to learn the temporal relations of the changes which continued in darkness. For this 20 white-enamelled pans each containing 10 prawns were placed in darkness on the day preceding the experiment. Between 6 and 7 in the morning these 20 groups of animals were each given a 5-second exposure to light at 250 ft. c. and then returned to darkness. At the end of 1, 5, 15, 30, and 60 minutes 4 of the 20 groups were given the 1-minute, 250 ft. c. test stimulus and thein returned again to darkness. The average state of the distal retinal pigment was determined for each of the 5 series at the time of the test-flash and at 30, 90, and 180 minutes thereafter. The results of this experiment are seen in figures 7 and 8 Here, it is clear that the effect of the 5-second conditioning flash in facilitating dark-

adaptation occurs gradually, building up to maximum facilitation of the dark-adapting process after about 30 minutes. With longer time in darkness, the effect of the facilitation gradually decreased again. Relatively little change has been wrought in the first 5 minutes in darknes. It is clear, then,

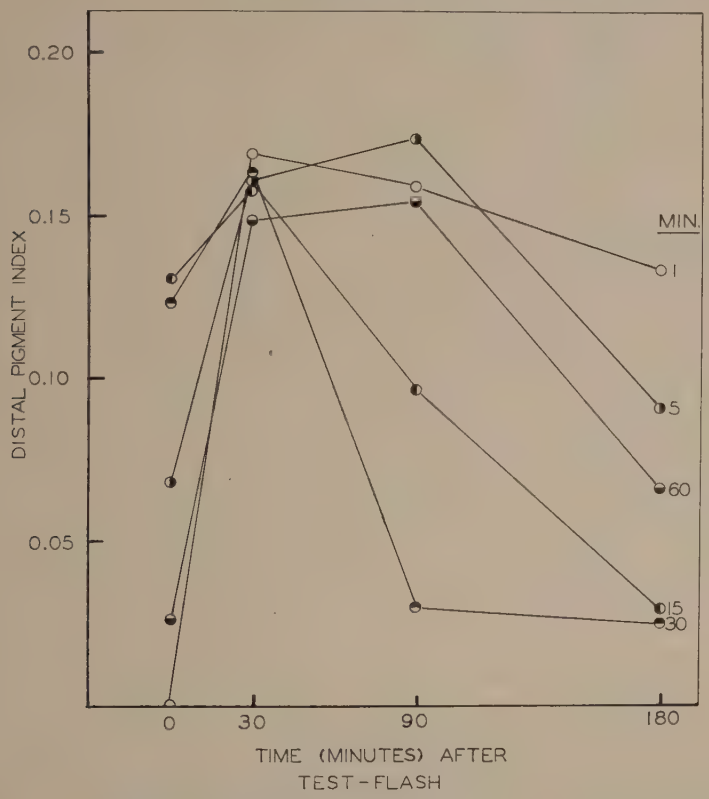

Fig. 7 Responses of overnight, dark-adapted prawns to a 1-minute, 250 ft. c. test flash following a 5-second, 250 ft. c. conditioning flash by different intervals of darkness.

that the change in physiological state necessary to induce the rapid subsequent dark-adaptation does not actually occur in the first 5 or 10 seconds of a 1-minute, 250 ft. c. flash. When the flash terminates at the end of 5 or 10 seconds, but not when it continues for a minute, the animals are in such a

state as to continue with such a process in the absence of bright-light stimulation.

Influence of "conditioning" stimuli applied after the test flash. In another type of experiment an attempt was made to learn whether 1-minute, 250 ft. c. flashes delivered to prawns

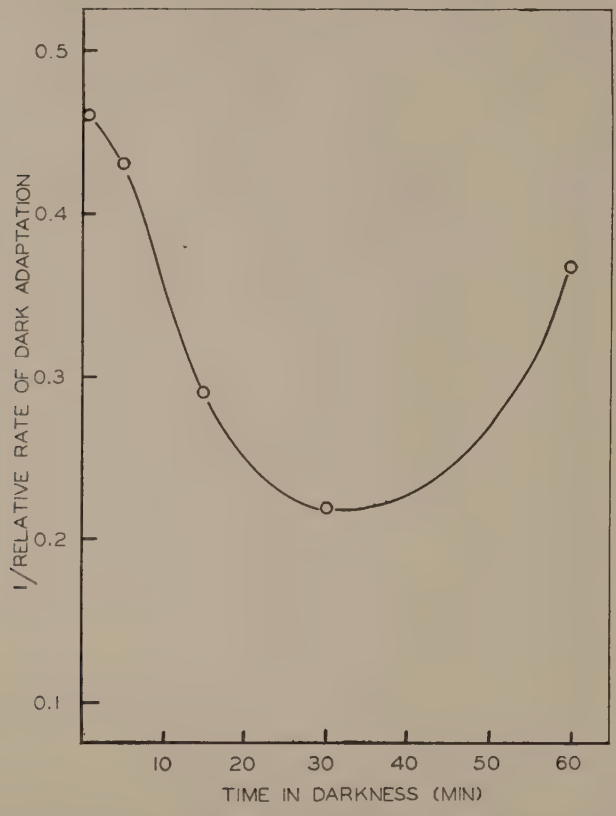

Fig. 8 The relationship between the reciprocal of dark-adapting facilitation and the time in darkness between a 5-second conditioning stimulus and the 1-minute, 250 ft. c. test flash.

when the distal retinal pigment was in different stages of light-adaptation following an earlier flash had different effects upon the facilitation of the dark-adapting process. Eleven groups of prawns, 10 in a group, which had since the preceding day been in complete darkness, were used. At about 9 A.M. all

11 groups were given the 250 ft. c., 1-minute flash and returned to darkness. After 30 minutes 5 of the groups were given a second 1-minute flash and at the same time the average distal pigment state of one of the groups in darkness was ascertained. The two groups, those animals which had received a single stimulus and those which had received two stimuli, a half hour apart, were then followed for 4 hours in darkness, their distal pigment state being determined after 30 minutes, 1, 2, 3, and 4 hours. The rate of dark-adaptation of both groups was very slow; the rate was characteristic of that of animals, at this time of day, which had had no conditioning stimulus prior to the test stimulus.

A final experiment was performed to learn whether conditioning stimuli of any type might possibly be effective at some time following rather than preceding the test stimulus, and that rapid dark-adaptation could be achieved in these animals which are quite refractory. For this 7 groups of 10 animals in white-enamelled pans were left in darkness from the preceding day. At about 5 A.M. all were given a 1-minute, 250 ft. c. flash. At 30 minutes, 1 and 2 hours sample groups were examined to determine the average state of the retinal pigment and to confirm that these animals were responding in a typical manner, exhibiting an inability to dark-adapt rapidly in the continuing darkness. At 9 A.M. 3 lots of 10 animals were removed from the darkroom and placed at a low illumination (about 0.1 ft. c.) that previous experiments in conditioning had indicated to result in subsequent rapid dark-adaptation in darkness. One group was returned to darkness after 15 minutes, another after 30 minutes, and the third after 60 minutes. One hour after each had been returned to darkness the average state of the distal retinal pigment was determined. The 7th group, serving as the control, was assayed at 10 A.M. The results of this experiment are illustrated in figure 9. There is no indication that the periods of low illumination at this time exerted any facilitating action of a dark-adapting character. The points for the three durations are dispersed randomly about the control.

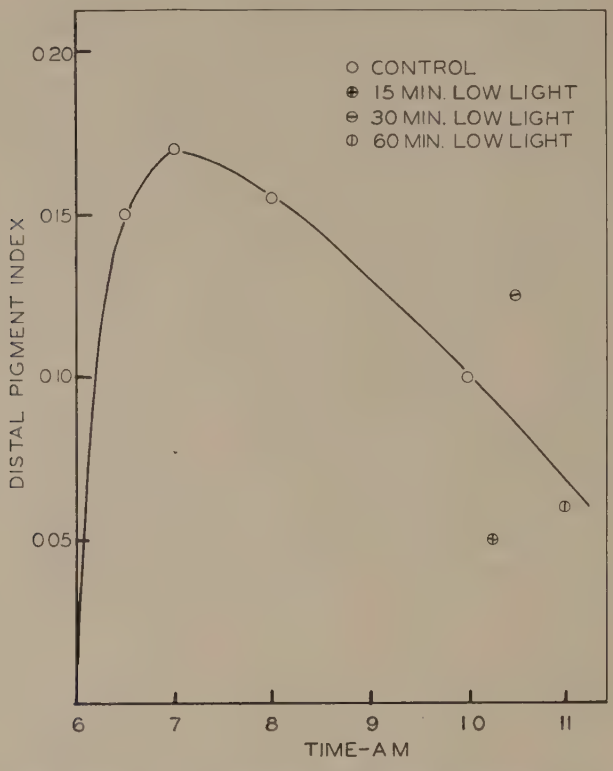

Fig. 9 The influence of low-intensity light stimuli delivered at 9 A.M. on the dark-adapting capacity of overnight, dark-adapted animals which had been given a 1-minute, 250 ft. c. test flash at 6 A.M.

DISCUSSION

Earlier work on the regulation of the distal retinal pigment of *Palaemonetes* has provided good evidence that this pigment is normally under the control of two blood-borne factors, a light-adapting and a dark-adapting principle. In terms of these, animals in continuous darkness have between 5 and 7 A.M. a high capacity to respond to bright light by the liberation of a light-adapting principle but have a very low capacity to respond to darkness by the liberation of a dark-adapting one. In the experiments reported here in which prawns were given low illumination conditioning stimuli for about half

an hour prior to a 1-minute, 250 ft. c. flash the animals developed a capacity to respond to darkness by the abundant liberation of a dark-adapting principle. The conditioning period could be (1) one in which the general response system of the animal becomes modified in such a manner that it calls for the liberation into the blood of the dark-adapting principle in response to darkness, or (2) a period in which the glands responsible for suppying dark-adapting material are actively synthesizing and storing a dark-adapting material, or (3) a period in which the blood-titre of dark-adapting principle is gradually being increased. In the last instance, it would become free to express itself only after the titre of light-adapting hormone produced in response to the test-flash had dropped sufficiently. At the moment it seems impossible to differentiate among these three alternatives.

When one considers the effects of brief 250 ft. c. flashes followed by darkness to complete a total conditioning period of 30 minutes, certain things appear quite evident. Up to 5 or 10 seconds of the conditioning light stimulus there is developed within the animals some state, which in the subsequent period of darkness, results in a continuing call for an increase in the capacity of the animal to dark-adapt when changed from light to darkness. Stimuli of these durations have relatively little activity in inducing continuing light-adaptation in the darkness which follows. On the other hand, as the light stimulus is applied for a longer time, there is a rapid inhibition of the preceding state and a substitution of another state which in the ensuing darkness calls chiefly for active liberation of light-adapting hormone. These alterations are represented diagrammatically in figure 10.

As the intensity of the conditioning illumination decreases the duration necessary to set up that physiological state in the animal to result chiefly in increasing its dark-adapting capacity or light-adapting capacity increases until some intensity, between 10 and 0.014 ft. c., is reached when the "light-adapting" physiological state is never reached irrespective of duration. These results appear to find their most rational

interpretation in terms of an hypothesis that in these dark-adapted animals a small total amount of light generates some sort of central nervous state which efferently calls chiefly for dark-adapting hormone when all stimuli are removed. A larger total amount of light generates a central nervous state that efferently calls chiefly or exclusively for light-adapting hormone when all stimuli are removed.

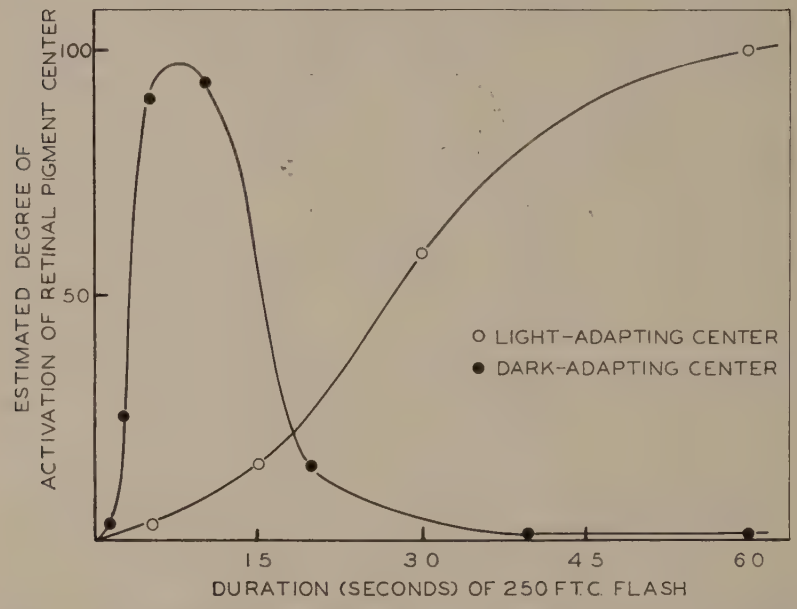

Fig. 10 Diagrammatic representation of an hypothesis of differential stimulation of dark- and light-adapting centers whose activities regulate the output of dark- and light-adapting hormones, respectively.

The results indicate that there is also involved a factor in which any given amount of light becomes less effective in producing its induced central state as longer times are required for it to summate. This would imply that the induced central state tends to decay slowly with time, as would reasonably be expected.

In figure 6, it will be noted that there is a secondary facilitation with time of the dark-adapting process. This, as the

abscissal scale units would indicate, is a relatively slow process. It is reasonable to presume that this is determined by the persistent diurnal rhythm in production of dark-adapting capacity since it was shown by Webb and Brown ('52) that there was, either in constant light or darkness, a rhythmically determined steady increase in dark-adapting capacity at this time of day. Also suggestive of this, is the fact that the 250, 50, and 10 ft. c. curves all tend eventually to converge in this trend.

The centers which are involved in these changes could well be those that are normally concerned in the precise regulation of the distal retinal pigment in adaptation to various illuminations (Sandeen and Brown, '52). These could well be the same centers which accurately regulate the relative amounts of the two hormones as a function of their state of excitation which is normally due to the light flux stimulating the photoreceptive material of the retinula cells. And any alteration in light intensity of the environment, or any hormonally induced alteration in the state of the distal retinal pigment, would consequently call forth compensatory hormonal activation within the body.

SUMMARY

1. *Palaemonetes* kept in constant darkness possesses very low ability to secrete dark-adapting hormone between 5 and 8 A.M.

2. On the other hand, prawns which have become light-adapted in response to normal dawn increase in illumination exhibit a high capacity for secreting dark-adapting hormone between 5 and 8 A.M.

3. It has been demonstrated that weak light stimulation, that is, high illumination for a very brief period, or low illumination for longer duration, generates a state within the prawns which results in elaboration chiefly of dark-adapting hormone.

4. Strong light stimulation generates a state within the animal which results in production exclusively of light-adapting hormone.

5. The physiological state induced by weak light stimulation, which calls for elaboration chiefly of dark-adapting hormone, requires many minutes to exert its action.

6. An hypothesis is set forth that the two endocrine sources regulating retinal pigment movements are in turn regulated by a "retinal pigment nervous center." This center shows graded activation by light stimulation, the degree of activity determining the quantities and proportions of the two retinal pigment hormones secreted.

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EFFECT OF AUREOMYCIN ON UTILIZATION OF PHYTOMONADS BY CILIATES

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Ciliates are commonly propagated on live food organisms such as *Tetrahymena* and bacteria (Doyle, '43). The use of phytomonads as food organisms is advantageous since they may be cultivated in a defined medium of a composition which does not meet the requirements for the growth of most bacteria. Since the phytomonads are dependent on simple acids or alcohols, rather than on carbohydrates, their growth may be controlled with respect to the ciliates by the expedient of omitting their special carbon source from the medium.

Several investigators have succeeded in growing *Perispira* (Dewey and Kidder, '40), *Kahlia* (Provasoli, '35), *Stylonychia* (Lilly, '42), *Pleurotricha* (Lilly, '42), and *Paramecia* (Johnson, '46) on phytomonads such as *Astasia, Euglena, Polytoma* and *Polytomella.* However, as pointed out by Dewey and Kidder ('40), and by Lilly ('42), the use of phytomonads as food organisms does not ordinarily give dependable results. While employing antibiotics for the purpose of freeing protozoan cultures of bacteria, we found that, under certain conditions, aureomycin stimulated the growth of ciliates on *Polytomella agilis.* With the aid of aureomycin we were able to cultivate *S. pustulata, Pl. lanceolata, P. aurelia, P. bursaria, P. caudatum,* and *P. multimicronucleatum* on this phytomonad, and also on *Euglena gracilis* and *E. gracilis var. bacillaris.*

Brown ('51) has reported a stimulative effect of aureomycin on *Tetrahymena* in pure culture. While it is possible that aureomycin may have a similar direct stimulative action on the ciliates reported in this paper, the growth promoting

action was found to be due chiefly to an ability of this antibiotic to affect the metabolism of phytomonads in a desirable way.

Medium employed

As reported previously (Little, Oleson and Williams, '51), our basal medium for cultivation of *P. agilis* consists of: 2 ml ethanol, 100 mg glutamine, 1 mg thiamine, 6 μg vitamin B_{12}, 0.5 gm Na_2HPO_4, 0.5 gm KH_2PO_4, 3.75 mg $MgSO_4 \cdot 7H_2O$, 20 mg $MgCl_2 \cdot 6H_2O$, 18.75 mg $CaCl_2 \cdot 2H_2O$, 0.3 mg $FeCl_3 \cdot 6H_2O$, 0.01 mg $MnCl_2 \cdot 4H_2O$, 0.01 mg $ZnCl_2$, and 1 liter H_2O; pH 6.8. The heat sterilized medium supports growth of *P. agilis, E. gracilis, E. gracilis var. bacillaris,* and *P. satura. P. satura* was found unsatisfactory as a food organism. Other phytomonads were not tested.

Conditions requiring aureomycin

Lilly ('42) pointed out that successful cultures of *S. pustulata* and *Pl. lanceolata* on *Euglena gracilis* and *Astasia klebsii* depend upon maintaining a proper balance between the populations of the ciliate and the food organism. We have found that the need for aureomycin as an aid to growth of the ciliates is greatest when the food organisms and the ciliates are introduced simultaneously into fresh medium. While such conditions give ample opportunity for changes to occur in the proportion of the two populations during growth, this does not seem to be the primary cause of failure of the ciliate to utilize the phytomonad in the absence of aureomycin. By a simple pH adjustment, fully grown cultures of phytomonads may be used as the starting point for cultivation of the ciliates in the absence of aureomycin.

Stylonychia and pleurotricha

Table 1 shows the stimulative effect of aureomycin on the growth of *S. pustulata* when this organism is introduced simultaneously with *P. agilis* into the basal medium. We added 10 ml of fully grown (5- to 6-day-old) *P. agilis* culture and

10 ml of fully grown (5- to 6-day-old) *S. pustulata* (maintained on *P. agilis*) to 500 ml of the basal medium. The cultures were mixed by rotation and periodic agitation and distributed in 10 ml volumes by burette to 6 by $\frac{5}{8}$ in. tubes containing small volumes of freshly prepared, sterile filtered antibiotic solution. The tubes were incubated at 25°C. in an air conditioned room. The growth response of the ciliate was determined by examining the tubes daily under low power magnification. The response reported is that obtained on the 6th day.

TABLE 1

Comparison of antibiotics for ability to aid growth of S. pustulata on P. agilis

ANTIBIOTIC		NO. POS. TOTAL TUBES	POS. TUBES CILIATES PER FIELD	EFFECT ON P. AGILIS ALONE	
				Final pH	Density reading
None		0/3	...	3.5	32
Aureomycin	1 μg/ml	2/3	6	6.5	36
	10 μg/ml	3/3	8	6.5	32
	50 μg/ml	3/3	8	6.6	25
Dihydrostreptomycin	10 μg/ml	0/3	...	6.6	25
	50 μg/ml	0/3	...	6.6	18
Chloromycetin	10 μg/ml	0/3	...	6.6	32
	50 μg/ml	0/3	...	6.6	18
Penicillin	10 μg/ml	0/3	...	6.6	30
	50 μg/ml	0/3	...	6.5	25

As shown in the table, aureomycin aids growth of *S. pustulata* on *P. agilis* whereas dihydrostreptomycin, chloromycetin and penicillin fail to give this effect. This experiment has been carried out a number of times with a strain of *S. pustulata* isolated by us and a strain of *Pl. lanceolata* obtained from Dr. D. M. Lilly. Usually, growth of these ciliates occurs in three to 4 days and is complete by the 6th day. The tubes are cleared of *P. agilis* to the extent that there is no turbidity attributable to the phytomonad, although a few viable cells of *P. agilis* do tend to persist. The cultures remain viable for about two weeks.

The tubes which fail to support growth of the ciliate invariably support growth of the food organism. Therefore, we have included in table 1 data on the effect of the antibiotics on the final pH and density of pure cultures of *P. agilis*. It will be noted that, in the absence of antibiotics, *P. agilis* produces a final pH of 3.5, whereas in the presence of the various antibiotics the pH remains around 6.5. The final pH of fully grown cultures of the ciliates has been found to be invariably around 7.5. While, as will be shown, fully grown cultures of *P. agilis* may be used as the starting point for cultivating the ciliates if the pH is adjusted to around 6.8, obviously the specificity of aureomycin cannot be accounted for entirely on the basis of inhibition of acid production. Comparison of density readings shows that dihydrostreptomycin and chloromycetin inhibit growth of *P. agilis* to some extent. It is interesting to note that the drop in pH after the growth of *P. agilis* is quite in contrast to the rise in pH which usually follows the growth of most phytomonads in tryptone-acetate and similar media which have been employed by many protozoologists. Hutner and Provasoli (Lwoff, '51) discuss the effect of antibiotics on phytomonads with respect to apochlorosis.

We have found that *Stylonychia* may be isolated readily from natural sources by the method of inoculating up to 1 ml volumes of sample simultaneously with 0.1 ml volumes of *P. agilis* into 10 ml volumes of the basal medium containing 50 μg per milliliter of aureomycin and 50 μg per milliliter of penicillin. Penicillin does not inhibit growth of the ciliates and the combination of the antibiotics is used in freeing the cultures of bacteria.

Paramecia

The paramecia do not grow when introduced simultaneously with *P. agilis* into the basal medium with or without aureomycin. Table 2 shows the stimulative effect of amino acids on the growth of *P. bursaria* under these conditions. We added 10 ml of fully grown (5- to 6-day-old) *P. agilis* culture and

10 ml of fully grown (5- to 6-day-old) *P. bursaria* maintained on *P. agilis* to 500 ml of the basal medium containing 50 μg per milliliter of aureomycin and distributed 10 ml volumes to tubes containing small volumes of heat sterilized supplements. The growth response was determined by examining the tubes daily. Growth was evident in three to 4 days and complete by the 6th day when positive tubes were cleared of *P. agilis,* and cultures remained viable for about two weeks.

TABLE 2

Effect of amino acids on growth of P. bursaria on P. agilis

SUPPLEMENT	AUREOMYCIN	NO. POS. TOTAL TUBES	POS. TUBES CILIATES PER FIELD
	μg/ml		
None	0	0/3	...
	50	0/3	...
Mixture of 21 amino acids (total 490 μg/ml)	0	0/3	...
	50	3/3	4
Mixture of 13 vitamins	50	0/3	...
Mixture of 12 miscellaneous	50	0/3	...
Glucose 100 μg/ml	50	0/3	...
DL-Aspartic acid 100 μg/ml	50	0/3	...
300 μg/ml	50	3/3	8
500 μg/ml	50	3/3	8
L-Glutamic acid 200 μg/ml	50	3/3	5
L-Histidine 200 μg/ml	50	3/3	4
Hydroxy-L-proline 200 μg/ml	50	3/3	6

This experiment has been carried out a number of times with strains of *P. bursaria* and *P. caudatum* isolated by us.

Johnson ('45) has shown that paramecia may be grown in proteose-peptone medium with supplements. Our first successful results on the growth of paramecia on *P. agilis* were obtained by use of a medium containing 21 amino acids, 13 vitamins, three carbohydrates, 12 miscellaneous substances, minerals and buffer. As shown in table 2, the amino acids were the only constituents of the more complex medium needed for growth of the paramecium on *P. agilis* in a medium designed for the phytomonad. Individual amino acids such as DL-

aspartic acid, L-glutamic acid, L-histidine, and hydroxy-L-proline were effective when used at 200 to 300 μg per milliliter. The effect of the amino acids appears to be non-specific. In addition to the amino acids, aureomycin was needed for growth of the paramecia when introduced simultaneously with *P. agilis* into the basal medium.

TABLE 3

Growth of ciliates on fully grown live P. agilis adjusted to pH 6.8

ORGANISM	AUREOMYCIN	NO. POS. TOTAL TUBES	POS. TUBES CILIATES PER FIELD	POS. TUBES FINAL pH
	μg/ml			
S. pustulata	0	3/3	10	7.6
	50	3/3	10	7.4
Pl. lanceolata	0	3/3	1	7.6
	50	3/3	7	7.3
P. aurelia	0	3/3	5	7.5
	50	3/3	7	7.3
P. bursaria	0	3/3	5	7.5
	50	3/3	5	7.3
P. caudatum	0	3/3	4	7.6
	50	3/3	5	7.3
P. multimicronucleatum	0	3/3	4	7.6
	50	3/3	5	7.3

Conditions not requiring aureomycin

Table 3 shows that a fully grown culture of *P. agilis* on the basal medium may be used as the starting point for cultivation of *S. pustulata, Pl. lanceolata, P. aurelia, P. bursaria, P. caudatum,* and *P. multimicronucleatum.* Since aureomycin is not needed under these conditions, the stimulative effect of the antibiotic when the ciliate and food organism are introduced simultaneously into fresh medium may be attributed to changes brought about in the metabolism of the phytomonad. A supplement of amino acids was not needed for growth of the paramecia on the fully grown culture of *P. agilis* on the basal medium.

P. agilis was grown for 5 to 6 days in 500 ml volumes of the basal medium. The cultures attained densities equivalent

to around 100,000 actively motile organisms per milliliter and a final pH of about 3.5. Since we were unable to obtain growth of the ciliates on *P. agilis* at this pH, the culture was adjusted to pH 6.8 with sterile Na_2HPO_4, using 1.7 to 2 ml of a 100 mg per milliliter solution of buffer per 500 ml of culture. The *P. agilis* culture was then dispensed with or without an aureomycin supplement and the tubes were seeded with 0.5 ml amounts of the ciliates, using three tubes on each. The growth responses followed the general pattern already described, except that the tubes were not always cleared as completely of *P. agiliis* in the absence of aureomycin as when aureomycin was used.

SUMMARY

Aureomycin was found to aid in the utilization of actively growing phytomonads by certain ciliates. The effect appeared to be due to an ability of this antibiotic to depress the metabolism of the phytomonads in a desirable way without inhibiting the ciliates. Dihydrostreptomycin, chloromycetin and penicillin were inactive. Methods are described for continuous propagation of *S. pustulata, Pl. lanceolata, P. aurelia, P. bursaria, P. caudatum,* and *P. multimicronucleatum* on *Polytomella agilis* in a defined medium designed for the phytomonad.

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ADAPTIVE ENZYME FORMATION IN RADIATION-SENSITIVE AND RADIATION-RESISTANT ESCHERICHIA COLI FOLLOWING EXPOSURE TO ULTRA-VIOLET

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THREE FIGURES

It has been established that individual strains of various bacteria including *Escherichia coli* may differ in their sensitivity to ultraviolet radiation (Rentschler and Nagy, '42). Using a radiation sensitive strain, Witkin ('47) was able to isolate radiation-resistant mutants designated B/r. These mutants arise from the sensitive strain B by spontaneous mutation at a rate of about 1×10^{-5}. The relatively greater resistance of strain B/r to ultraviolet radiation and x-rays extends to numerous other agents, including chemicals elevating the redox potential of culture media (Bryson, '49, '53).

Doses of ultraviolet sterilizing 99.9% of the cells in a population of strain B may sterilize only 50% of similarly treated B/r cultures. In addition, strain B and B/r are reported to differ in nucleic acid content (Morse and Carter, '49; Marshak, '51). Since ultraviolet radiation is strongly absorbed by protein and nucleoprotein components of the cell, it was considered desirable to study the effect of lethal ultraviolet doses on various enzyme systems of *E. coli*, with particular attention to adaptive enzyme formation and the extent of differences between B and B/r as measured by

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comparative oxidative activity following irradiation. The term "adaptive" is used only to define an enzyme system showing significant lag in the onset of maximum activity, without implications as to the enzyme level of the unadapted cell.

MATERIALS AND METHODS

Two strains of *E. coli* were employed — the radiation-sensitive strain B and a radiation-resistant B/r obtained from Dr. Witkin. Unless otherwise stated, cells were grown in M9 medium (ammonium medium, Anderson, '46 [2]) under forced aeration for 18–20 hours, centrifuged, resuspended in saline, then divided into two equal portions. One portion was retained as the untreated control, the other receiving different doses of irradiation at 6 ergs/mm^2/sec. The source of radiation was a GE T-15 mercury vapor lamp with reflector, placed at 30 inches from the culture. Irradiation was performed by placing 7 ml of cell suspensions in petri plates and agitating the dish during exposure to minimize screening. All cells were then recentrifuged and washed twice with M/15 phosphate buffer (pH 6.8). A Klett-Summerson colorimeter was used to adjust washed suspension to the same turbidity both before and after irradiation, insuring approximately the same number of cells in each Warburg vessel. Assays were made to determine viable cells in both treated and untreated final suspensions by plating suitable dilutions on nutrient agar. One milliliter of the cell preparation containing a total cell number of approximately 9×10^9 was then pipetted into a Warburg vessel containing 1 ml of M/15 phosphate buffer and 0.5 ml of distilled H_2O. One-half milliliter of .06 M substrate in the sidearm was tipped into the main vessel after an equilibration period of 10 minutes.

EXPERIMENTAL RESULTS

Before beginning a study of radiation effects on adaptive enzyme systems, it appeared essential for comparison pur-

[2] Na_2HPO_4 misprinted in reference as $NaHPO_4$.

poses to examine the effects of UV treatment on enzymes of the so-called constitutive type. Oxidation of glucose followed manometrically with the Warburg was chosen as the first system to investigate. It was found that suspensions of B and B/r irradiated from 90 sec. to 180 sec. showed essentially

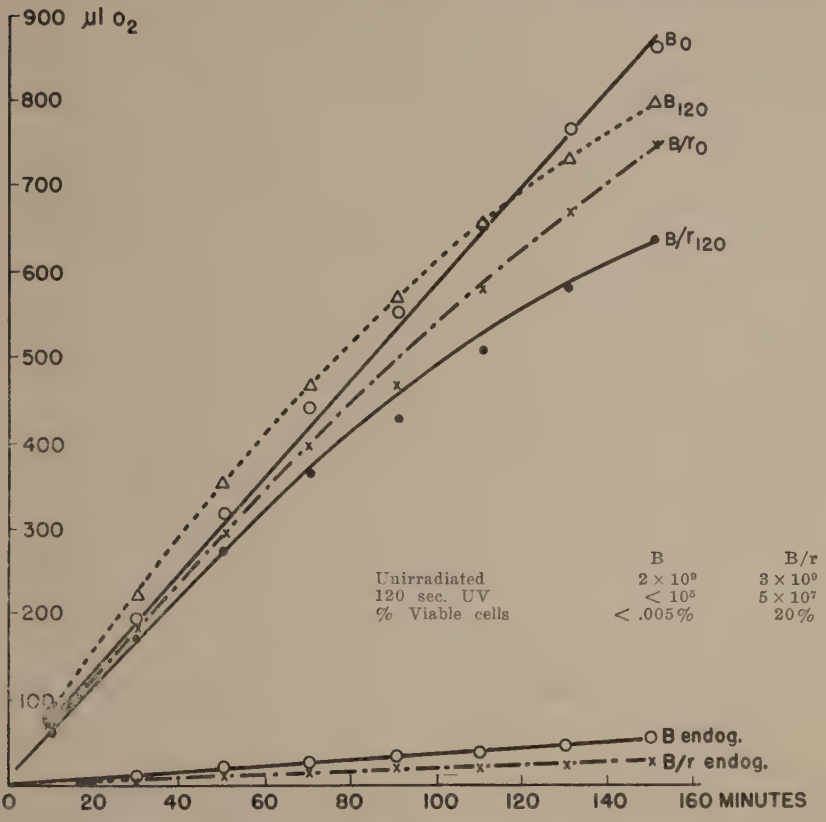


Fig. 1 Comparison of irradiated and unirradiated cells of strains B and B/r; viability and O_2 uptake on glucose.

the same O_2 uptake on glucose as the untreated controls for 60 minutes. Twenty per cent inactivation has been the most observed; generally the rates are almost identical. From figure 1 it may be seen that no marked change occurs in the rates of O_2 uptake following irradiation for 120 sec. (720

ergs/mm^2), employing either strain B or B/r. The tabular data of figure 1 show that the viable fraction of B/r after ultraviolet treatment is 200 times that of strain B. At least 99% of each strain is no longer able to form visible colonies if assayed for viability, yet this does not affect respiratory activity on glucose for at least one hour. These results suggest that the sterilizing action of ultraviolet may not occur until considerable cell metabolism has taken place, in conformity with microscopic evidence of limited residual growth following radiation damage.

Influence of population size on adaptation. Attention was now directed to adaptive systems. In a study of the enzyme activity of predominantly inviable cells it is essential to know the potential contribution of any small viable residue remaining in the population after treatment, particularly if opportunity exists for the few viable cells to multiply during the experiment. Control populations were therefore established consisting of numbers of unirradiated cells corresponding in titer to the viable component remaining after more concentrated suspensions are exposed to ultraviolet. It was noted that 4×10^6 cells/ml of strain B will take up less than 30 µl of oxygen in eight hours on a galactose substrate, even in the presence of nitrogen. Yet irradiated suspensions of 2×10^8 cells/ml containing 5×10^6 viable cells took up 1000 µl of oxygen in less than three hours. Evidently enzyme activity is not due to the viable fraction, as will be further established by a comparison of strains B and B/r.

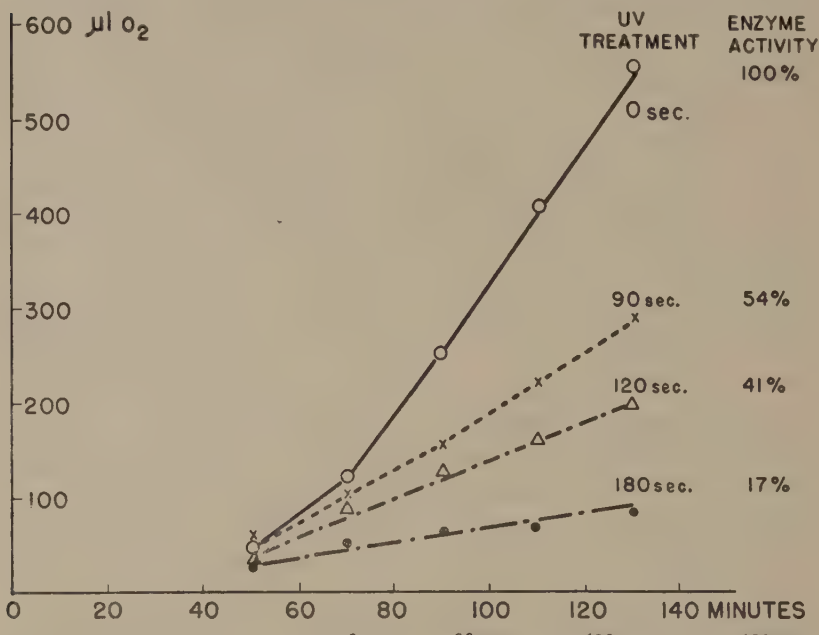
An unforeseen influence of population density on adaptation was noted during experiments on galactose utilization. If an initially high concentration of washed cells of a strain B culture (1.8×10^9/ml) is diluted to one-half and irradiated for 90 seconds (6 ergs/mm^2/sec.), the activity on galactose will be approximately 55% of the control. When the original cell suspension containing the undiluted number of cells is irradiated, activity of the suspension is about 90% of the original. Viability in both cases is reduced to less than 0.1%, and the actual number of viable cells does not seem to be a

determinative factor in this observation. For example, it was noted that strain B/r, at an original concentration of 1.3×10^9 cells, loses about 50% of its adaptation activity at a UV dose of 90 seconds although the viable count is 20% of the original.

From these and other similar observations, it would seem then that one variable — concentration of cells at time of irradiation — heretofore not considered in the experimental procedure, can greatly influence the inhibition of adaptation. That this inhibition is independent of viable cell number at the end of the UV treatment is clearly borne out by the fact that identical results can be obtained with strains B and B/r. Apparently then, the protein synthesizing system involved in adaptation to galactose by *E. coli* can be protected from inhibition by UV by increasing the cell numbers at time of irradiation. This protective effect does not extend to the cell division apparatus, for apparently death (as determined by viable count) will occur to almost the same extent. There exist perhaps more particles in the concentrated cell suspension which will combine with some of the UV products that would ordinarily damage the adaptation system. The damaging effects of UV on cell division and on adaptation seem to be qualitatively different.

Effect of exogenous nitrogen on rate and degree of adaptation. The first experiments performed with lactose, arabinose or galactose as adaptive substrates were carried out using cell suspensions without any exogenous source of nitrogen. According to Spiegelman ('50) yeast cells can adapt to the substrate in the absence of exogenous nitrogen, but the level of enzymatic activity attained and the rate at which it is reached is considerably increased in the presence of a nitrogen source. Monod ('50) claims that for *E. coli,* adaptation is observed at a reduced rate in the absence of nitrogen. The results obtained in this laboratory illustrate that with *E. coli* B/r, adaptation to arabinose and lactose can occur with resting cells in the absence of exogenous nitrogen; however, the degree of adaptation is considerably increased, as with yeast cells,

if nitrogen is added to the system. Viable counts indicate that one division, at most, takes place when 170 μg/ml (.02%) NH_4NO_3 is present, while the respiration rate on galactose after adaptation occurs is 10 times greater than in the induction period. Without nitrogen the rate of O_2 consumption on arabinose increases about three times. Irradiation of 1080

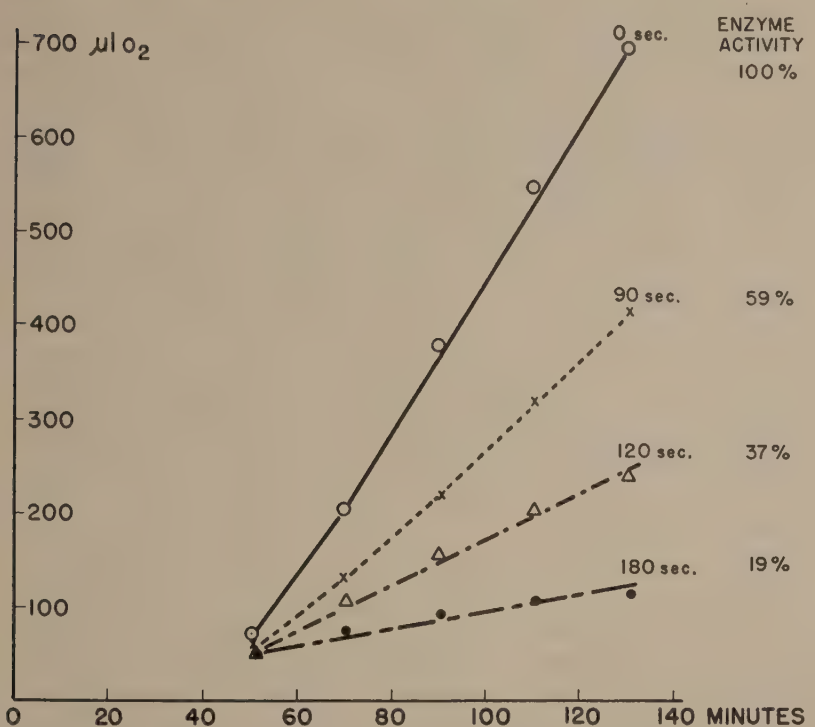


	0	90 sec.	120 sec.	180 sec.
No. or % cells viable	1.7×10^9	.006%	<.0006%	<.00006%
% enzyme activity	100%	54%	41%	17%

Fig. 2 Influence of UV irradiation on galactozymase activity of *E. coli* strain B.

ergs/mm^2 (180 sec.) is sufficient to retard adaptation to lactose, arabinose and galactose in the presence of NH_4NO_3 for at least three hours. At this dose, the viable count is about 2×10^6 cells, indicating that all but 1% of the population is unable to reproduce, even though the whole population is able to oxidize such sugars as glucose and fructose as measured manometrically. The small number of viable cells is apparently unable to synthesize the galactozymase system within three hours' contact with the substrate.

Further comparison of galactose utilization by strains B and B/r following ultraviolet radiation. Using .02% NH_4Cl and purified galactose, a more complete comparison was made of the O_2 uptake shown by strains of B and B/r. The results are shown in figures 2 and 3. Upon comparing the relative viability of B and B/r after exposure to radiation, the two



	0	*90 sec.*	*120 sec.*	*180 sec.*
No. or % cells viable	1.5×10^9	26%	8.6%	0.3%
% enzyme activity	100%	59%	37%	19%

Fig. 3 Influence of UV irradiation on galactozymase activity of *E. coli* strain B/r.

strains are found to differ. In contrast, enzymatic activity is approximately equal in the radiation-sensitive and radiation-resistant strains as seen in the figures. In both strains, enzyme formation is equally and progressively depressed by increasing doses of ultraviolet radiation. Brandt, Freeman and Swensen ('51) found no inhibition of galactozymase when *S.*

cerevisiae was treated with x-rays at doses giving comparable survival levels.

DISCUSSION

While this investigation was in progress (Bryson, Kaplan and Rosenblum, '51) it was reported by Swenson and Giese ('51) that x-rays at low doses have little effect on the ability of yeast to form adaptive enzymes. More recently, Billen and Lichtstein ('52) have studied the influence of x-ray exposure on the metabolism of *E. coli,* finding that high quantities of radiation were required to interfere with the adaptive formation of formic hydrogenlyase. The result of our research is in general agreement with the conclusion that certain enzyme systems are not damaged by doses of radiation sufficient to interfere seriously with normal cell multiplication. Even the very early research on ultraviolet effects showed that cells incapable of normal division as a result of radiation damage may nevertheless continue to synthesize cellular constituents, forming lengthy "snakes" or filaments (Gates, '33). The radiation-resistant strain B/r will form microcolonies after exposure to levels of ultraviolet that induce snaking in strain B.

Recent investigations indicate that this adaptation phenomenon is a result of synthetic processes. Azide, 2,4-dinitrophenol, and arsenate, agents which interfere with the oxidative phosphorylation of intermediates, resulting in energy loss for the cell, also prevent adaptive enzyme formation (Spiegelman, '50). It would seem, then, that ultraviolet acts generally to destroy synthetic mechanisms of the resting cell as well as the division mechanism itself. The fact that with constitutive enzyme systems (glucose, fructose, sucrose) we have observed almost no inhibition of O_2 consumption from irradiated samples, while with the several adaptive systems we have tested activity was absent after 180 seconds' irradiation, suggests that ultraviolet may not interfere with existing enzyme systems, but only with those needing the energy generated by oxidative phosphorylation for enzyme synthesis.

A comparison of the amount (if any) of oxidative assimilation occurring from utilization of glucose after irradiation, coupled with an investigation of the amount of inorganic phosphate esterified would serve perhaps to elucidate the specific means by which synthetic processes are inhibited by ultraviolet (or products of ultraviolet). Other enzyme systems known to be able to operate without a special source of energy must be tested as well.

Swenson ('50) has shown that the action spectrum of the inhibition of galactozymase production of yeast cells by ultraviolet light is identical to the absorption spectrum of nucleoprotein to ultraviolet. This evidence would seem to support such a hypothesis, and would be expected in consideration of the fact that the nucleoprotein of the cells is closely associated with synthetic activities.

CONCLUSIONS

E. coli made inviable by exposure to ultraviolet radiation (1080 ergs/mm^2) retain the normal capacity to utilize glucose. Oxygen uptake on adaptive substrates such as galactose is progressively reduced as a result of increasing doses of radiation, but reduction in enzymatic activity does not parallel loss of viability. Thus in strain B, 85% of adaptive activity may remain after more than 99% of the cells are sterilized. Although strains B and B/r differ significantly in viability following radiation damage they are identical in oxygen uptake on substrates presumed to require adaptive enzyme formation. Therefore, differences between strain B and B/r in sensitivity of the mechanism of cell division to radiation damage do not extend to include the synthesis of adaptive enzymes.

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RESPIRATION, RHODANESE, AND GROWTH IN ESCHERICHIA COLI

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FOUR FIGURES

Respiration has been studied in many organisms under numerous experimental conditions. In general, certain relatively constant respiratory rate levels have been observed. However, in some instances not levels but cyclical changes have appeared. Zeuthen ('49) measured periodic changes in oxygen uptake of developing eggs, where the rate of respiration increases during the time of cell division.

In this paper, the author will also describe an endogenous respiration in Escherichia coli whose rate is periodic under suitable conditions. Not only is there a periodicity in respiration, but also in the activity of an enzyme, rhodanese. These cyclical events have been correlated with the period of cell division under the conditions of this experiment.

Rhodanese was used as an assay enzyme mainly because of other work which was in progress involving some of its characteristics. As a brief introduction, rhodanese is an enzyme which, in vitro, converts thiosulphate plus cyanide ions to thiocyanate. It was discovered by Lang ('33), who extracted it from cow liver, and measured its activity in homogenates. It is present in greatest concentration in liver and kidneys of vertebrates, and according to Borei (unpublished), its presence is scattered throughout the animal kingdom. Lang ('33) found the enzyme in Escherichia coli, and the author has confirmed his results.

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METHODS AND MATERIALS

Escherichia coli, strain 16, was obtained from the Molteno Institute of Cambridge University, and was maintained in pure culture on nutrient agar, new sub-cultures being made monthly. The bacteria which were used in these experiments were subsequently transferred to Schlesinger's Lactate medium, 500 cm^3 in 100 Ehrlemeyer flasks, aerated under sterile conditions, at 37°. Using large inoculums, harvests were made between 10 and 15 hours after the time of inoculation. The bacteria were harvested in a preparatory Collatz-(Ecco)-centrifuge at 2900 r.p.m., and were subsequently washed in 100 ml of 0.9% NaCl. They were recentrifuged at 17,000 r.p.m. in an International Refrigerated Centrifuge for 5 minutes at plus 2°. Washing was repeated once. These were the bacteria which were used in the following experiments.

Rhodanese activity. Rhodanese activity was measured in the following manner: To 2 ml of 0.2 molar borate buffer (H_3BO_4 plus NaOH) plus 0.36 ml of 1/1 solution of $Na_2S_2O_3$ (0.1 molar) and KCN (0.1 molar), 0.2 ml of bacterial suspension was added. The final pH was 9.0. The bacteria were incubated in this solution for 30 minutes at room temperature without shaking, and the reaction was stopped with the addition of 2.5 ml ferric nitrate plus nitric acid (50 gm $Fe(NO_3)9H_2O$ plus 100 ml of 65% HNO_3 in 1 l of distilled H_2O). A red-colored iron-thiocyanate complex results. The reaction mixture was centrifuged in an International Cold Centrifuge at 17,000 r.p.m. for 5 minutes, and the optical density was measured in a Spekker photometer (filter 602, 1 cm cells). All optical density readings were taken within 12 hours after the test.

Oxygen uptake. Oxygen uptake was measured in Warburg flasks, at 25°, using the Warburg direct method, absorbing CO_2 in 0.4 ml 15% KOH, with filter paper in the center well. The flasks contained 3 ml of bacterial suspension (7 mg/ml in 0.9% NaCl, being resuspended in fresh NaCl). Rhodanese activity was measured in these bacterial suspensions.

Cell growth. Cell growth was measured in cultures maintained at 25° in a constant temperature incubator. The cells were grown in Schlesinger's Lactate medium, with aeration, from inocula that had received previous anaerobic conditioning. Inoculation masses were adjusted so that the culture might start well beyond the lag phase. Cell division was estimated by measuring changes in optical density (Spekker photometer, filter 608, 1 cm cells). The density readings were converted to dry-weight values with an appropriate calibration curve. That density changes in the logarithmic phase of growth are a good estimate of cell division has been shown by Monod ('42). All density readings were made during the logarithmic phase.

Preconditioning of bacteria. Preconditioning was accomplished by placing in Thunberg tubes the thick bacterial suspensions harvested from the International Centrifuge. These tubes were evacuated with a water-pump and filled with gases under atmospheric pressure. The bacterial suspensions (5 ml of 5 mg/ml bacteria in 0.9% NaCl) were then incubated at 37° for 5 hours with shaking. All the bacteria used in these experiments received this preconditioning treatment except the respiratory control which received no preconditioning.

RESULTS

The rhodanese activity in washed non-proliferating suspensions which have had previous anaerobic incubation in nitrogen is periodic. This may be seen from figure 1, curves 1 and 2, which represent two different experiments. The average time between minima is approximately one and one-half hours, the rhythm ceasing at approximately 7 hours. At this time, the periodicity ceases and the activity drops at a relatively rapid rate.

From figure 2, curves 1 and 2, the Q_{O_2} 30 minutes (uptake of oxygen in mm^3/mg dry tissue/30 minutes) has the same periodicity as the rhodanese activity in *different* experiments on cells which have received previous anaerobic incubation. In all these experiments, no significant change in turbidity could

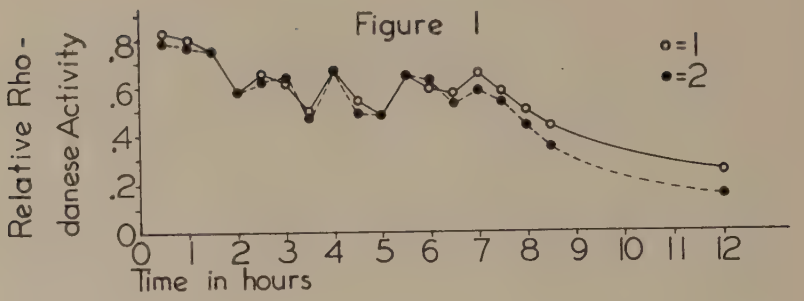
Figure 1
Relative Rhodanese Activity
o=1
•=2
Time in hours

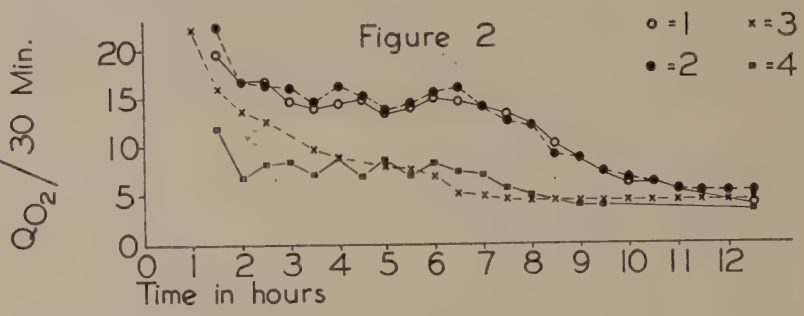
Figure 2
Q_{O_2}/30 Min.
o=1
x=3
•=2
▪=4
Time in hours

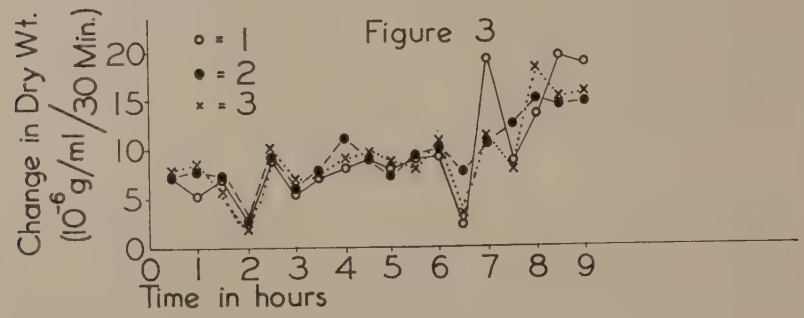
Figure 3
Change in Dry Wt. (10^{-6} g/ml/30 Min.)
o=1
•=2
x=3
Time in hours

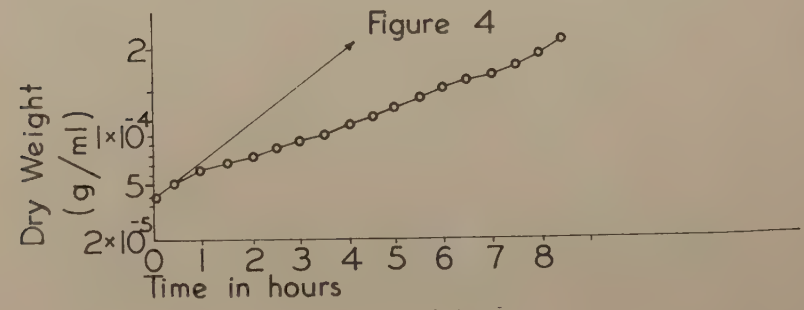
Figure 4
Dry Weight (g/ml)
Time in hours

Figures 1–4

be observed from the beginning to the end of each experiment. There are minima at 2, 3½, and 5 hours, the average period between minima being 1½ hours. Beyond 6 hours there is a difference in rhythm between the two curves which may be due to a loss of synchronization. At approximately 7½ hours, the periodicity ceases and the oxygen uptake decreases at a relatively rapid rate until 10 hours, where it levels off.

From figure 2, curve 3, a culture which is allowed to respire freely, with no previous anaerobic conditioning, shows no periodicity. The decrease in respiration is logarithmic, and converges with the oxygen-uptake curve of the anaerobically incubated bacteria at approximately 11 hours.

From figure 2, curve 4, bacteria which have been incubated in oxygen in a Thunberg tube during the same period as the anaerobically incubated bacteria have a much reduced respiration, but the same periodicity. The oxygen must be utilized well before the end of the incubation period, the anaerobic state then being maintained for the remainder of the incubation. There are minima at 2, 3½, 5, and 6½ hours, corresponding to an average period of 1½ hours. The respiration declines steadily after 7 hours with no further periodicity.

From figure 3, curves 1, 2, and 3, representing three different cultures, the change in dry weight/30 minutes in a lactate culture of Escherichia coli is periodic, with an inoculum previously incubated anaerobically. Minima which are common to all three curves may be seen at 2, 3, 5, 6½, and 8 hours, the average period between minima being 1.4 hours.

From figure 4, if dry weight vs. time is plotted in the usual manner (log of dry weight vs. time), the experimenter would probably interpret deviations from a straight line as scatter. However, scatter seems improbable since the deviations are synchronous from culture to culture (5 experiments in triplicate), and since the periodicity is the same as found with rhodanese and oxygen uptake in washed cultures. The mean generation time as calculated from the initial growth

rate (the arrow), is approximately one and one-half hours, which corresponds rather closely with the average period between successive minima.

The figures shown are from typical experiments. In 5 additional experiments not presented here, the same general phenomena were observed — a periodicity of approximately one and one-half hours in rhodanese activity, cell division, and oxygen uptake.

It has been found that a long period of anaerobic incubation is not necessary for synchronization, but, since the observed phenomena are reproducible at this incubation time, there seemed to be no reason for changing the methods used. The same phenomenon was observed with incubation times of two hours.

DISCUSSION

It seems that anaerobic incubation of Escherichia coli is sufficient to synchronize bacterial respiration in a washed culture, rhodanese activity in a washed culture, and cell division in a proliferating culture. That this is indeed synchronization, is evident from the following reasoning.

The variables measured represent the activities of millions of bacteria. All the curves shown are periodic with respect to time, except the respiration curve of bacteria which have received no previous anaerobic incubation, and the same periodicity is observed in all experiments. During the time of the experiment, all variables which can change the rate of cell metabolism are controlled and negligible. Therefore, changes in oxygen uptake, rhodanese activity, and proliferation rate must reflect intrinsic changes in the cell's metabolism. Now, if these changes in cell metabolism are random (the metabolism of one cell being independent of that of another cell in the culture), then measurements of a given variable in the entire culture would be a result of an averaging effect which would reduce all fluctuations from some mean value to zero. However, the fluctuations are not zero, and assuming the foregoing conditions, the most reasonable explanation of these results is that the bacteria are syn-

chronized and that intrinsic changes in bacterial metabolism are being studied.

The anaerobic incubation of bacteria at 37° evidently does not depress the level of bacterial metabolism to any great extent. If one compares the respiration curves of bacteria which have been synchronized by the anaerobic pre-treatment with those which have not been pre-treated, the initial respiratory rate of the pre-treated ones is as high as in those which have not been pre-treated, and furthermore remains high for a considerable period of time. It is true that synchronization will accentuate periods of high respiration, but since the minima do not approach zero, it is probable that a considerable number of bacteria are respiring. The pre-treatment in one sense may be considered as a state of metabolic rest. Readily oxidizable substrates may build up during this time, for after again being transferred to an aerobic environment, the level of oxidation remains high for 7½ hours. This is not the case in the suspension without pre-treatment which drops steadily from zero time. Increased metabolic activity after incubation is also shown by negligible lag in the growth curves.

It is apparent why the generation time should be calculated from the initial growth rate when compared with the rate of oxygen-uptake and rhodanese activity. For after anaerobic pre-treatment it is in the beginning when the substrates necessary for oxidative metabolism and also cell division are high. After a cell goes through a division, it is conceivable that it would take a longer period of time on the average to synthesize sufficient metabolites before it can undergo another division.

If the periodicities in the three phenomena are causally connected, certain conclusions may be considered. First, respiration increases at the time of cell divsion, as has been found by Zeuthen ('49) in developing eggs of the frog, *Rana platyrrhina* and the worm, *Urechis caupo*. Second, in a washed suspension, where there is little proliferation, the bacterial cell is evidently geared metabolically to the period of cell

division. The cell's interior may be kinetically in a state of flux, with processes continuing to operate which would normally culminate in a cell division when the proper environmental conditions are present. Finally, if rhodanese activity is indeed coupled to the level of aerobic oxidation, it may be indicative of general enzyme turnover, even under "resting" conditions.

SUMMARY

1. A periodic endogenous respiration and rhodanese activity is observed in washed cultures of Escherichia coli which have been anaerobically pre-treated for 5 hours at 37°.

2. This periodicity corresponds to the initial generation time, when these bacteria are placed in a medium suitable for growth.

3. The results are discussed, and implications are suggested regarding a possible revision of the concept of a "resting" bacterial cells.

ACKNOWLEDGMENT

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